Neurod6 and *Neurod1* are required for the survival of a novel subset of midbrain dopaminergic neurons projecting to the lateral septum

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Declaration

I **Shabana Khan** confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Midbrain dopaminergic neurons are highly heterogeneous. They differ in their target and afferent projections and firing patterns, and therefore in their functional properties. The molecular underpinnings of this heterogeneity are largely unknown and there is a paucity of markers that distinguish these functional subsets. This thesis reports the identification and characterisation of a novel subset of midbrain dopaminergic neurons located in the ventral tegmental area that expresses the basic helix-loop-helix transcription factor, Neurogenic Differentiation Factor-6 (NEUROD6). Retrograde fluorogold tracing experiments demonstrate that *Neurod6*+ midbrain dopaminergic neurons project to two distinct septal regions, the dorsal lateral and intermediate region of the lateral septum. Loss-of-function studies in mice demonstrate that *Neurod6* and the closely related family member *Neurod1* are both specifically required for the survival of this lateral-septum projecting neuronal subset during development. These findings underscore the complex organisation of midbrain dopaminergic neurons and provide an entry point for future studies of the functions of the *Neurod6*+ subset of midbrain dopaminergic neurons.

Significance Statement

Midbrain dopaminergic neurons regulate diverse brain functions, including voluntary movement and cognitive and emotive behaviours. These neurons are heterogeneous and distinct subsets are thought to regulate different behaviours. However, we currently lack the means to identify and modify gene function in specific subsets of midbrain dopaminergic neurons. The work presented here identifies the

transcription factor NEUROD6 as a specific marker for a novel subset of midbrain dopaminergic neurons in the ventral midbrain that project to the lateral septum and reveals essential roles for *Neurod1* and *Neurod6* in the survival of these neurons during development. My findings highlight the molecular and anatomical heterogeneity of midbrain dopaminergic neurons and contribute to a better understanding of this functionally complex group of neurons.

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"To him who observes [scientists with artistic hobbies] from afar, it appears as though they are scattering and dissipating their energies, while in reality, they are channeling and strengthening them…The investigator would possess something of this happy combination of attributes: an artistic temperament which impels him to search for, and have the admiration of, the number, beauty, and harmony of things."

—Santiago Ramón y Cajal

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Abbreviations

AADC - Aromatic-l-Amino-Acid Decarboxylase

AAV - Adeno-Associated Virus

Adcyap - Adenylyl Cyclase Activating Peptide

ADHD - Attention-Deficit Hyperactivity Disorder

AHPs - Afterhyperpolarisations

Aldh1a1 - Aldehyde Dehydrogenase Family 1

Amg - Amygdala

AMPA - α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid

AMPH - Amphetamine

AP - Action Potential

AU - Arbitrary Units

BDA - Biotin Dextran Amine

BDNF - Brain-Derived Neurotrophic Factor

bHLH - basic Helix-Loop-Helix

BSA - Bovine Serum Albumin

cAMP - cyclic adenosine monophosphate

CB - Calbindin-D28K

cKO - Conditional Knockout

CR - Calretinin

Crx - Cone- Rod Homeobox

CVG - Cochlear-Vestibular Ganglion

DA - Dopamine

DAT - Dopamine Transporter

DEPC - Diethylpyrocarbonate

dPN - dorsal paranigral nucleus

DRG - Dorsal Root Ganglia

E - Embryonic day

ERK1/2 - Extracellular-Signal-Regulated Kinase 1/2

ES - Embryonic Stem

FG - Fluorogold

FGF8 - Fibroblast Growth Factor 8

FP - Floor Plate

GABA - γ-Aminobutyric Acid

GAD - glutamate decarboxylase

GAP-43 - Growth Associated Protein 43

Gbx2 - Gastrulation Brain Homeobox 2

GFP - Green Fluorescent Protein

GIRK2 - G-Protein Coupled Inwardly Rectifying K Channel 2

GLP-1R - Glucagon-Like Peptide 1 Receptor

GPCRs - G-protein coupled receptors

Gpr83 - G-protein coupled receptor 83

Grp - Gastrin Releasing Peptide

HCl - Hydrochloric Acid

HRP - Horseradish Peroxidase

IF - Interfascicular Nucleus

Igfbp4 - Insulin Growth Factor Binding Protein 4

IPN - Interpeduncular Nucleus

IsO - Isthmic Organiser

IZ - Intermediate Zone

K+ - Potassium

LCM - Laser Capture Microdissection

Lpl - Lipoprotein Lipase

LS - Lateral Septum

LSd - Dorsal Lateral Septum

LSi - Intermediate Lateral Septum

LSN - Lateral Septal Nucleus

LSv - ventral Lateral Septum

mDA - Midbrain Dopamine/ Midbrain Dopaminergic

MS - Medial Septum

MZ - Mantle Zone

NA - Noradrenaline

NAc - Nucleus Accumbens

NBF - Neutral Buffered Formalin

NCAM - Neural Cell Adhesion Molecule

NEUROD1 - Neuronal Differentiation Factor 1

NEUROD6 - Neuronal Differentiation Factor-6

NGF - Nerve Growth Factor

NL - Nuclear Localisation

Npn1 - Neuropilin 1

Npn2 - Neuropilin 2

OCT - Optimum Cutting Temperature

OT - Olfactory Tubercle

Otx2 - Orthodenticle Homeobox 2

p75NTR - Pan-Neurotrophin Receptor Gene

PBP - Parabrachial Nucleus

PBS - Phosphate Buffered Saline

PD - Parkinson's Disease

PFC - Prefrontal Cortex

Pitx3 - Paired-like homeodomain 3

PKA - protein kinase A

PN - Paranigral Nucleus

Prg1 - Plasticity Related Gene 1

PZ/ VZ - Proliferative Ventricular Zone

RA - Retinoic Acid

ROIs - Regions Of Interest

RP - Roof Plate

RRF - Retrorubral Field

RT - Room Temperature

Shh - Sonic Hedgehog

shRNA - Short Hairpin RNA

SNc - Substantia Nigra Pars Compacta

SNl - Substantia Nigra Pars Lateralis

SNr - Substantia Nigra Pars Reticulata

Str - Striatum

Strep-HRP - Streptavidin-Horse Raddish Peroxidase

TEA - Trietahnolamine

TH - Tyrosine Hydroxylase

TrkB - Tyrosine Kinase B

TUNEL - Transferase-Mediated Biotinylated Utp Nick-End Labelling

VIP - Vasoactive Intestinal Peptide

VMAT2 - Vesicular Monoamine Transporter 2

vPN - ventral Paranigral Nucleus

VS - Ventral Septum

VTA - Ventral Tegmental Area

VZ - Ventricular Zone

Wnt1 - Wnt Family Member 1

WT - Wild-Type

Chapter 1. Introduction

1.1 Short History of Dopamine

Dopamine (DA) was initially synthesised in the early 1900s (Barger and Ewins, 1910; Mannich and Jacobsohn, 1910) however this particular catecholamine did not become of much interest to scientists until later in the 1950s. DA was for the first time detected in mammalian tissues by Goodall in 1951 where it was found in the heart and adrenal medulla of sheep (Goodall, 1951). Up until that point, DA was still referred to as 3-hydroxytyramine, which was the short form for its proper chemical name β-3,4-Dihydroxyphenethylamine and in 1952, Sir Henry Dale coined the name "dopamine" (Blaschko, 1952). During this time the idea of DA having its own unique biological actions, that may be different from those of other better studied catecholamines at the time such as adrenaline and noradrenaline and aside from DA's established role in their synthesis, was not given much attention. The identification of dopamine as a neurotransmitter in the brain and not merely just a precursor of noradrenaline was demonstrated by the Swedish pharmacologist, Arvid Carlsson, in 1957. His work at the time included developing an assay to measure dopamine in the brain where he made the observation that the basal ganglia was where the highest regional concentration of DA existed (Hornykiewicz, 2008). This led on to his experiments on reserpine, an antipsychotic that due to its effects on depletion of DA, also produced loss of DA motor control similar to the motor impairment observed in clinical features of Parkinsonism. Carlsson went on to show that the DA precursor, L-dopa, was effective in the treatment of motor symptoms of Parkinsonism and is still considered today as one of the main drug treatments for Parkinson's Disease (PD) patients. Aside from PD, he is also famous for his

'dopamine theory of schizophrenia' documenting the role of DA in the development of extra-pyramidal side effects of antipsychotic medications. Carlsson and co-recipients Eric Kandel and Paul Greengard, won the Nobel Prize in Physiology or Medicine in the year 2000 for their revolutionary work on dopamine.

1.2 Dopamine Synthesis

Catecholamines are monoamines which are organic compounds that have a benzene with 2 hydroxyl groups at carbons 1 and 2 and a side chain amine. Three catecholamines exist in nature; adrenaline, noradrenaline (NA) and DA, which are all derived from the amino acid tyrosine. DA synthesis in nervous system tissues is a two-step process. Firstly, tyrosine is converted into DOPA in a dehydration process catalysed by the enzyme tyrosine hydroxylase (TH). This is the rate-limiting step in DA synthesis and uses tetrahydrobiopterin and molecular oxygen. Secondly, the decarboxylation of L-DOPA by aromatic-L-amino-acid decarboxylase (AADC) into DA. In the brain, DA has several functions in facilitating movement, motivation, reward and addictive behaviours.

1.3 Dopamine Signaling

In the brain, endogenous neurotransmitter DA can act as a ligand by binding to DA receptors and modulating downstream DA signalling. DA receptors are metabotropic G-protein coupled receptors (GPCRs) and are broadly divided into two main subtypes; the D_1-like class and D_2-like class of DA receptors (Beaulieu and Gainetdinov, 2011).

The D_1-like family of DA receptor subtypes includes D_1 and D_5, which are excitatory in action since their activation is coupled to Gαs which activates adenylyl cyclase. This leads to an increase in the concentration of the second messenger cyclic adenosine monophosphate (cAMP) resulting in protein kinase A (PKA) activity. This DA receptor subtype is uniquely expressed on DA-target postsynaptic cells (Beaulieu and Gainetdinov, 2011).

In contrast, the D_2-like family of DA receptor subtypes includes D_2, D_3 (Sokoloff *et al*., 2006) and D_4 (Rondou *et al*., 2010), which are inhibitory in action since their activation is coupled to Gαi which inhibits adenylyl cyclase. This results in a decrease in the concentration of cAMP leading to reduced PKA activity (Beaulieu and Gainetdinov, 2011). Also, unlike the D_1-class, the D_2 and D_3 receptors are expressed both on postsynaptic DA-target cells and presynaptically on DA-producing neurons (Sokoloff *et al*., 2006; Rankin *et al*., 2010; Rondou *et al*., 2010). The levels, broad expression patterns and various functions of D_1 – D_5 receptors in the brain are summarised in Appendix 7.1.

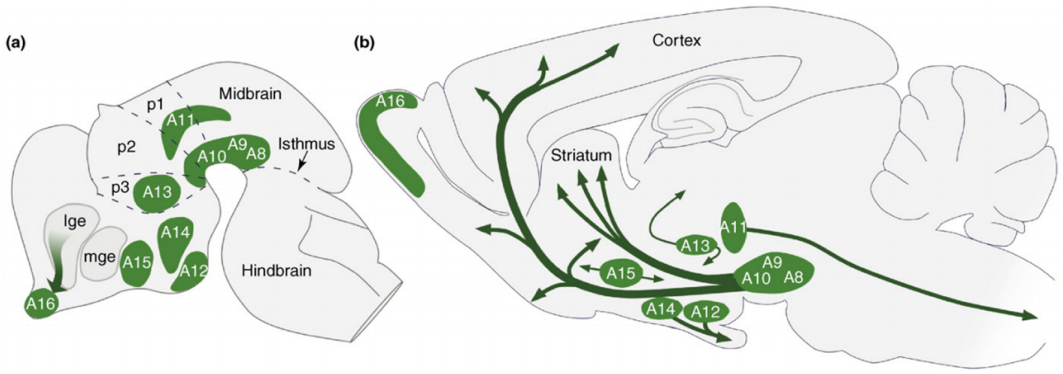

Figure 1-1. Distribution of DA groups in the developing embryonic brain and in the adult brain of rodents (cited from Bjorklund and Dunnet, 2007)

A total of 9 DA groups which include the A8- A16 DA populations within the (a) developing embryonic brain and (b) adult brain of rodents. In the adult, these DA groups project their axons to a wide range of targets within the brain. 75% of neurons that use DA as their primary neurotransmitter are located within the midbrain A8, A9 and A10 subpopulation nuclei corresponding to the retrorubral field (RRF), substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA) respectively. These midbrain dopamine (mDA) neurons project their axons to regions within the forebrain including multiple cortical and striatal regions.

1.4 The origins of midbrain dopamine neurons

In the developing embryonic and adult brain, a total of 9 DA groups exist (Figure 1-1). These are classified as the A8 – A16 DA groups (Figure 1-1). In the adult brain, 75% of total neurons that primarily use dopamine as their neurotransmitter, are located within the ventral midbrain within three primary midbrain dopamine (mDA) nuclei (Ang, 2006; Wallen and Perlmann, 2003). These mDA subpopulations include the A8 retrorubral field (RRF), the A9 substantia nigra pars compacta (SNc) and the A10 ventral tegmental area (VTA) mDA subpopulations (Figure 1-2; Stott and Ang, 2013). These midbrain dopaminergic (mDA) neurons express the rate-limiting enzyme TH involved in DA synthesis (Figure 1-2; Stott and Ang, 2013). These mDA neurons represent a large heterogenous mixed population that differ in their anatomical location, cellular

morphology, physiological properties, axonal projections, forebrain targets and consequently in their underlying functions. Degeneration of SNc neurons gives rise to PD whereas dysfunction of VTA neurons is implicated in several neuropsychiatric disorders. This chapter will highlight both the development of these mDA neurons and their anatomical, cellular, molecular, physiological and functional heterogeneity.

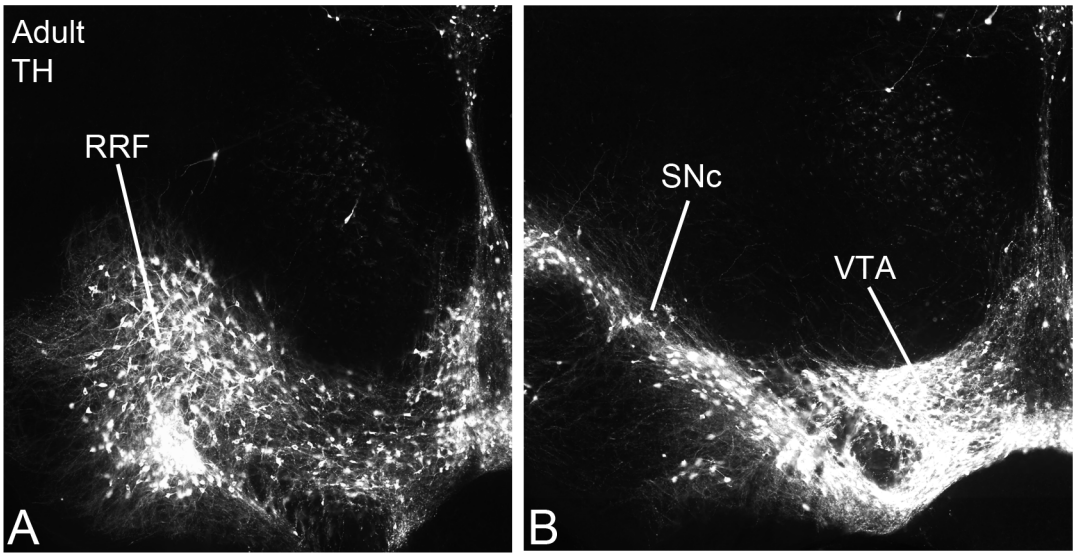

Figure 1-2 The mDA subpopulations: (A8) RRF, (A9) SNc and (A10) VTA
Immunohistological fluorescence staining for visualisation of mDA neurons using an antibody for the rate- limiting enzyme TH on a (A) Coronal section of the adult mouse brain at the level of the retrorubral field (RRF) and on a (B) Coronal section of the adult mouse brain at the level of the substantia nigra (SNc) and ventral tegmental area (VTA) mDA subpopulations.

1.5 mDA neuron development

Neural subtype specification begins with the subdivision of the rostral CNS into broad territories; the forebrain, midbrain and hindbrain. These broad regions of the brain are patterned along the anterior – posterior and dorsal – ventral axis by signalling factors that are secreted from distinct organising centres. These secreted signals in

turn activate the expression of specific transcription factors that are required for the specification of progenitors into specific neuronal fates whilst tightly regulating their proliferation to ensure the generation of precise pool of progenitors. Specific sets of transcription factors are expressed in a combinatorial manner across the various stages of mDA neuronal development. Firstly, an early differentiation stage where mDA progenitor cells differentiate into immature mDA neurons. Secondly, the late differentiation stage where immature mDA neurons differentiate further into mature mDA neurons. And finally the maintenance and survival of these mature mDA neurons from the embryo to adult stages.

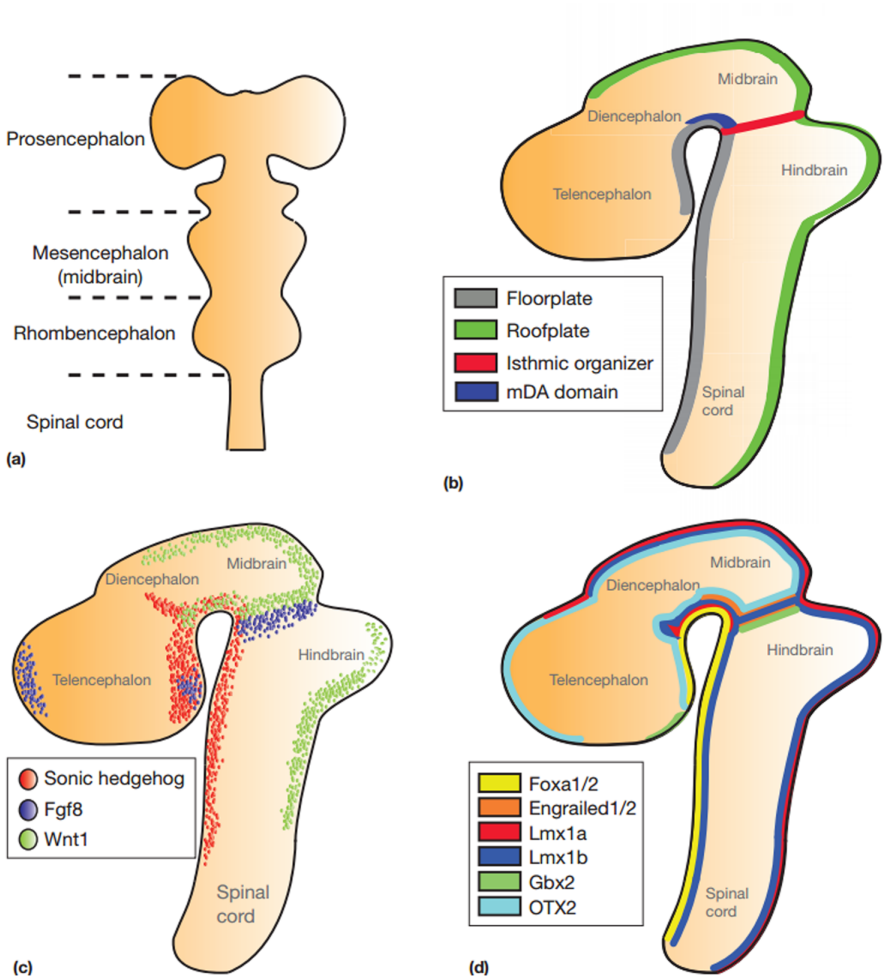

Figure 1-3 Early development of the mDA domain relies on signalling centres, secreted proteins and several transcription factors (cited from Stott and Ang, 2013)

(a) Schematic represents the developing anterior end of the neural tube divided into the prosencepahlon, mesencephalon, rhombencephalon and the spinal cord. The midbrain arises from the mesencephalon, (b) The mDA domain is located at the ventral curve of the midbrain region in mice at embryonic (E) day E10 and its development is influenced by the anatomical location and secretory factors released from the three main mDA organising centres which include the floor plate (FP), the roof plate (RP) and the Isthmic Organiser (IsO), (c) Secretory proteins are released from each of these areas including Sonic hedgehog (Shh) from the FP, FGF8 from the IsO and Wnt1 secreted from multiple areas, (d) Secreted proteins from the organising centres then regulate the expression of several transcription factors within different midbrain areas for regional specification and induction of mDA progenitor cell development.

1.5.1 Early Midbrain Patterning & Regional Specification of mDA progenitors

Neuralisation of the ectoderm during gastrulation gives rise to the 'neural plate' also known as the neuropepithelium. The neural plate folds along its length and the fusing of its lateral edges form the dorsal midline of a 'neural tube' (Copp *et al*., 2003). Closing of the neural tube allows for the regionalisation of the brain by the formation of three vesicles at the anterior end of the embryo; the prosencephalon, mesencephalon, rhombencephalon and spinal cord (Stott and Ang, 2013; Figure 1-3a). Prosoncephalon (forebrain) gives rise to the telencephalon (future cerebral cortex, basal ganglia, internal capsule, insular cortex, lateral ventricles and corpus callosum) and diencephalon (future thalamus, epithalamus, subthalamus and hypothalamus). The Rhombencephalon (hindbrain) gives rise to the metencephalon (future cerebellum and pons) and the myelencephalon (future high and low medulla). The midbrain (superior and inferior colliculi) arises from the mesencephalon, which is the structure that lies between the prosoncephalon and the rhombencephalon in the developing neural tube.

The regional specification of the mDA domain relies on an important organising centre called the Isthmic organiser or Isthmus (IsO) located at the boundary between mesencephalon-metencephalon border also known as the midbrain-hindbrain boundary (Figure 1-3b). The orthodenticle homeobox 2 (Otx2) and gastrulation brain homeobox 2 (Gbx2), are two homeodomain transcription factors that are required for establishing the correct positioning and maintenance of the IsO by mutual repression. The IsO is formed at the posterior extent of the Otx2 expression boundary and the anterior limit of Gbx2 expression (Ang, 2006; Figure 1-3). Loss- of function studies of Otx2 and Gbx2 reveal a forward and backward shift of the midbrain/ hindbrain boundary, respectively (Broccoli *et al*., 1999; Millet *et al*., 1999) highlighting the fundamental roles of Otx2 and Gbx2 in IsO maintenance and midbrain patterning.

The midbrain contains two other signalling centres; the floor plate (FP) along the ventral midbrain midline and the roof plate (RP) which runs along the dorsal midline of the midbrain (Figure 1-3b). Fibroblast growth factor 8 (FGF8) proteins are secreted from the IsO, sonic hedgehog (Shh) from the floor plate and Wnt1 from many signalling centres (Stott and Ang, 2013; Figure 1-3b,c). Together, these secretory proteins are required for the induction of mDA neurons before E9.5 by regulating the expression of downstream transcription factors within the midbrain including the paired box genes *Pax2/5/8*, engrailed homeobox genes *En1/2* and the LIM homeobox gene *Lmx1b* (Guo *et al*., 2007; Figure 1-3d).

Members of the forkhead box family of transcription factors, Foxa1 and Foxa2, regulate many phases of mDA neurogenesis in a dose-dependent manner (Ferri *et al*., 2007). Specifically, Foxa1/2 control the specification of mDA progenitors by

regulating the expression of downstream genes including *Ngn2*, *Nurr1*, *En1*, *Aadc* and *Th* (Ferri *et al*., 2007) during both early- and late- differentiation phases of mDA neurons.

1.5.2 mDA Differentiation & Maturation

Birthdating studies in mice have revealed that between E9.5 and E13.5, mDA progenitors exit the cell cycle and undergo an early differentiation phase to generate post-mitotic immature mDA neurons (Bayer *et al*., 1995). Transcription factors expressed in mDA progenitor cells that facilitate the neuronal differentiation of mDA progenitors into differentiating immature mDA neurons include Ngn2 (Andersson *et al*., 2006a), Msx1 (Andersson *et al*., 2006b). Mash1 is also expressed in mDA progenitors but is not required for their differentiation however is compensatory for Ngn2 function in mDA differentiation (Kele *et al*., 2006).

Immature mDA neurons migrate ventrally along radial glia fibers (Shults *et al*., 1990; Kawano *et al*., 1995) from the proliferative ventricular zone (VZ) of the ventral midbrain to the intermediate zone (IZ). Here, immature mDA neurons begin expression of the immature mDA marker nuclear receptor related 1 (Nurr1; Zetterstrom *et al*., 1997). Nurr1 is expressed in both immature and mature mDA neurons and is required for expression of late- differentiation markers, levels of which are diminished in Nurr1 conditional mutants (Kadkhodaei *et al*., 2009).

The expression of BIII-tubulin, a general neuronal marker, is also initiated at this differentiation stage to maintained in mature mDA neurons (Kele *et al*., 2006).

While migrating along radial glia, immature mDA neurons undergo a late differentiation step and transition from the IZ into the mantle zone (MZ) where in addition to expressing the immature markers, they also begin expressing mature

mDA markers including AADC, PITX3 and TH (Smidt *et al*., 2004). At this stage while mDA neurons are undergoing late differentiation from immature to mature states, the onset of AADC (Ang, 2006) facilitates the conversion of L-DOPA to DA, which is then synthesised in mDA neurons during maturation. Other mature mDA markers also include the vesicular monoamine transporter 2 (VMAT2) and dopamine transporter (DAT). VMAT2 is an internal membrane protein that packages DA from the cytosol into synaptic vesicles for vesicular release of DA and other co-neurotransmitters such as GABA. DAT is a membrane-spanning protein that pumps DA already released in the synapse back into the cytosol of mDA cells for storage before release.

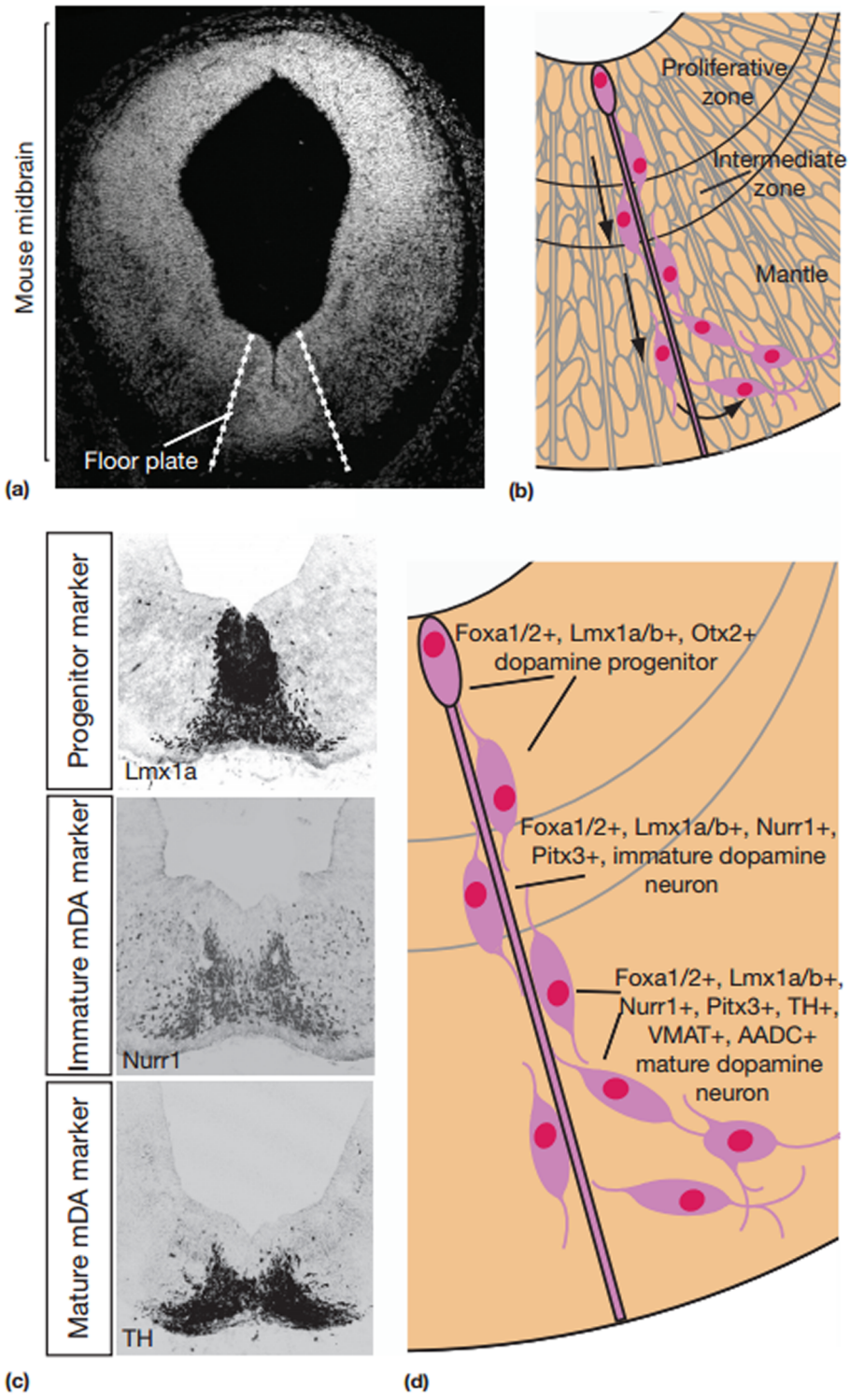

Figure 1-4 Stages of mDA differentiation and migration along FP during embryonic ventral midbrain development (cited from Stott and Ang, 2013)

(a) Coronal section at the level of the ventral midbrain in E12 mouse embryonic brain exposing the FP region which gives rise to mDA progenitors in the **(b)** proliferative ventricular zone (PZ/ VZ) which then migrate along shafts of radial glia

during their early differentiation stage into immature mDA neurons into the intermediate zone (IZ) and then late differentiation stage into mature mDA neurons reaching the mantle zone (MZ). **(c)** Lmx1a is expressed early in mDA progenitor cells and serves as an mDA progenitor marker in the VZ/PZ, Nurr1 is a marker for immature mDA neurons and TH is a marker for mature mDA neurons. **(d)** Transcriptional control in mDA progenitors expressing Otx2, Foxa1/2 and Lmx1a/b which then undergo early differentiation into immature mDA neurons while migrating into the IZ. Here they continue to express Foxa1/2 and Lmx1a/b that are required at multiple stages of mDA neuron development. Immature mDA neurons begin to express Nurr1 and Pitx3. Late differentiation step of immature mDA neurons occurs while they migrate into the MZ and here mature mDA neurons express in addition to previous markers, TH, VMAT and AADC.

1.5.3 Maintenance & Survival of mDA neurons

The expression of many of the genes implicated in the generation of mDA neurons are also maintained in the adult such as *Foxa2*, *Lmx1a/b*, *En1/2* and *Nurr1*. Mutant analyses have revealed that apart from their role in the development of mDA neurons, these factors also play critical functions in the maintenance and survival of mature mDA neurons. Analysis of mice heterozygous for *Foxa2*, revealed late-onset mDA neuron degeneration resulting in motor impairments in these mice (Kittappa *et al*., 2007). This study highlighted the role of *Foxa2* in the maintenance and survival of mature mDA neurons. A recent study by Doucet-Beaupre *et al*., (2016) also demonstrated the role for *Lmx1a/b* genes in the survival of mature mDA neurons in adult mice via the regulation of mitochondrial functions that are independent from their early role in mDA neuron development. Inactivation of *Lmx1a/b* recapitulated cellular pathogenic features observed in PD including α-synuclein aggregation and degeneration of mDA neurons (Doucet-Beaupre *et al*., 2016). The underlying neurodegenerative mechanism caused by the loss of Lmx1a/b was mitochondrial dysfunction measured by compromised respiratory chain activity hence increased oxidative stress and mitochondrial DNA damage (Doucet-Beaupre *et al*., 2016).

In addition to *Lmx1a/b*, *En1/2* genes also function in mDA neuron maintenance and survival via regulation of the sensitivity of these neurons to mitochondrial insult in a cell autonomous and gene-dosage dependent manner (Alavian *et al*., 2009). Alavian *et al*. (2009) analysed both homozygous and heterozygous *En1/2* mutants. *En1/2* homozygous mice died at birth resulting in a complete loss of mDA neurons by mid-gestation and an overall deletion of the midbrain and rostral hindbrain (Alavian *et al*., 2009). Viable *En1/2* heterozygous mice however survive but postnatally they exhibit a progressive degeneration of mDA neurons resulting in PD-like molecular hallmarks and behavioural abnormalities (Alavian *et al*., 2009). In these En1/2 mutants, cell death of mDA neurons occurs due to increased expression of the pan-neurotrophin receptor gene (p75NTR) and a reduction of extracellular-signal-regulated kinase 1/2 (Erk1/2) activity (Alavian *et al*., 2009). Due to these effects, Engrailed expression is also reduced and mitochondrial stability is compromised by inhibition of the anti-apoptotic members of the Bcl-2 protein family (Alavian *et al*., 2009).

Late functions of Nurr1 in the maintenance of maturing and mature mDA neurons of adult mice were also studied by conditional targeting of the *Nurr1* gene in mice (Kadkhodaei *et al*., 2009). Conditional Nurr1 mice allowed for controlling the ablation of Nurr1 in either (i) late stage of mDA neuron development, while the mDA neurons are transitioning from their immature to mature stage, by using cNurr1^{DATCre} mice (generated by crossing conditional Nurr1 mice with Cre under the control of DAT (DATCre) mice) and (ii) in the adult brain once the mDA neurons have already acquired their mature state by using transduction of adeno-associated virus (AAV) Cre-encoding vectors (cNurr1^{AAVCre}) (Kadkhodaei *et al*., 2009).

During mDA maturation, analysis of cNurr1^{DATCre} revealed a rapid loss of striatal dopamine content, sudden loss of mDA neuron marker expression and degeneration of mDA neurons (Kadkhodaei *et al*., 2009).

Inactivation of Nurr1 in already matured mDA neurons in adult cNurr1AAVCre-injected mouse brains however revealed a more progressive and slower loss striatal DA and reduction in mDA neuron marker expression (Kadkhodaei *et al*., 2009). Together, these results suggest the additional crucial and important roles for developmental transcription factors in terminally differentiated mature mDA neurons in the maintenance of their mDA properties, mitochondrial functions and ultimately their survival.

The paired-like homeodomain transcription factor 3 (Pitx3) belongs to the RIEG/PITX homeobox family, is expressed in post-mitotic mDA neurons which is maintained into adulthood (Smidt *et al*., 1997). Despite, Pitx3 expression in all mDA neurons, the SNc and VTA subpopulations have differential requirements for Pitx3 function (Maxwell *et al*., 2005). Overexpression studies of Pitx3 in human non-neuronal and mouse embryonic stem cell lines demonstrated Pitx3's ability to induce endogenous expression of TH (Messmer *et al*., 2007). Further evidence to support the requirement for Pitx3 in mDA neuronal survival is from the analysis of the naturally occurring *Pitx3* mutant mice known as the 'aphakia' mouse. This mouse model has two 5' deletions in the *Pitx3* gene (Rieger *et al*., 2001). These *Pitx3*$^{ak/ak}$ mice display significant reduction of mDA axons in the dorsal striatum (Nunes *et al*., 2003) and present with motor impairments due to a significant loss of mDA neurons in the SNc (Hwang *et al*., 2003). Analysis of transgenic *Pitx3*-GFP knock-in mice, in which the entire *Pitx3* coding sequence is replaced with the green fluorescent protein (GFP)

reporter under the control of the *Pitx3* promoter, revealed that selective loss of SNc neurons compared to the VTA is due to the dependency for Pitx3- induced TH expression in SNc neurons but not in VTA neurons (Maxwell *et al*., 2005). Since at E12.5, *Pitx3* expression in laterally positioned early SNc immature mDA neurons is initiated before the onset of TH expression (Maxwell *et al*., 2005). In contrast, medially positioned VTA immature mDA neurons already expressed TH ahead of *Pitx3* expression suggesting that TH expression is not induced by *Pitx3* in this subpopulation (Maxwell *et al*., 2005).

In addition to inducing the expression of TH exclusively in SNc mDA neurons (Messmer *et al*., 2007; Maxwell *et al*., 2005), Pitx3 regulates other downstream targets within SNc neurons including the neurotrophic factors BDNF and GDNF (Peng *et al*., 2011) and the gene encoding the enzyme ALDH1A1 (Chung *et al*., 2005b). Together, these results highlight the role of PITX3 in the survival of SNc subpopulation of mDA neurons while also highlighting the heterogeneity of mDA neurons and their requirements for different transcription factor proteins.

Besides these broadly expressed genes that are expressed in mDA neurons in early development and in mature mDA neurons, the survival of post-mitotic, terminally differentiated, mature mDA neurons is further controlled at a more subset-specific level. Specific genes are expressed less broadly and more specifically in only a porportional ‘subset’ of mDA neurons compared to the entire population, this can include either SNc-specific or VTA-specific subsets, ranging from 95% of each of the SNc or VTA subpopulations or down to less than 10% of the mDA domain. Subset-specific genes may not only be required for mDA survival but may also be important for the correct circuitry and topography of mDA neurons. In light of this, heterogeneity

of mDA neurons is highly complex and other factors that are important for the survival of mDA neurons, specifically VTA-specific subsets include Otx2, Calbindin, Aldh1a1 (see later) and Neurod6, which is the subject of this thesis.

1.6 Heterogeneity of mDA neurons

1.6.1 mDA cell anatomical location & morphology

Histofluorescence techniques have identified the anatomical mapping and localisation of mDA neurons. This historical experimental approach has traditionally divided mDA neurons in to three distinct subpopulations; the lateral A9 SNc mDA neurons and the mDA neurons of the medial A10 VTA (Figure 1-2b) as well as A8 RRF mDA neurons (Figure 1-2a). Majority of mDA neurons lie within these regions but small numbers of mDA neurons are also localised to the substantia nigra pars reticulata (SNr) and substantia nigra pars lateralis (SNl; Bjorklund and Dunnet, 2007). SNc neurons are also further sub-divided into a dorsal and ventral tier according to their cell morphology and connectivity pattern. Dorsal tier SNc neurons have a rounded and fusiform shape, and exhibit relatively low expression of dopamine transporter (DAT). SNc neurons of the ventral tier are form a densely packed sheet. These are more angular in shape, are calbindin-negative, have higher DAT expression levels and express the G-protein coupled inwardly rectifying K channel 2 (GIRK2) ion channel protein (Bjorklund and Dunnet, 2007). VTA-specific mDA neurons are localised within the interfascicular nucleus (IF), paranigral nucleus (PN), and parabrachial nucleus (PBP); Figure 4-1l).

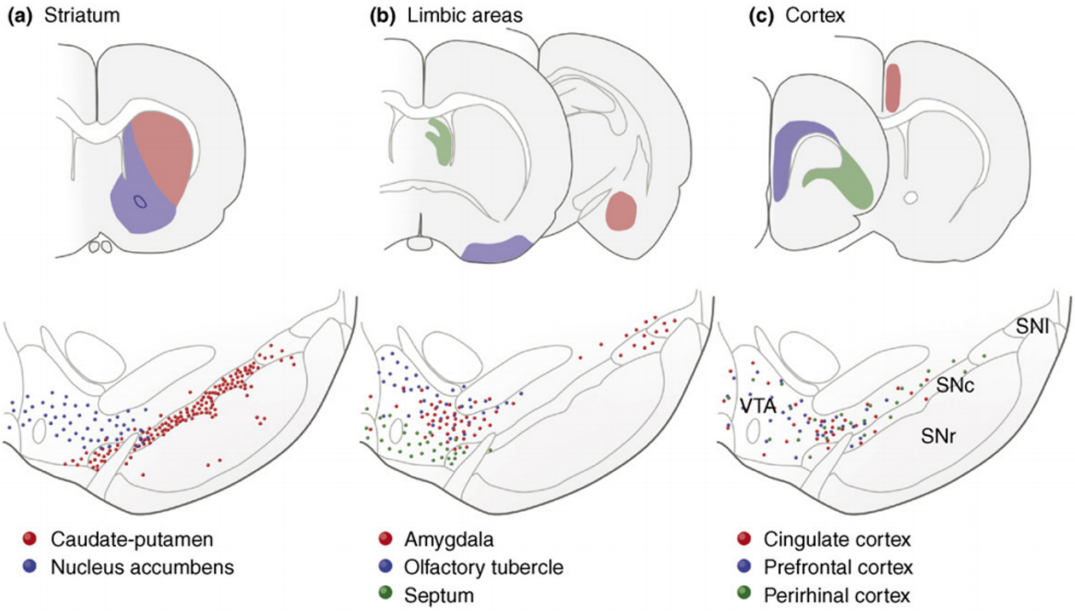

Figure 1-5 Heterogeneity of mDA neurons in the ventral midbrain and their projection pathways in the forebrain of rat (cited from Bjorklund and Dunnett, 2007).

(a) The anatomical location of mDA neurons that send their axons along the mesostriatal pathway is mainly within the SNc and innervate the caudate-putamen of the dorsal striatum. This pathway also consists of mDA neurons in the VTA that innervate the ventral nucleus accumbens (NAc) regions, **(b)** mDA neurons that project their axons along the mesolimbic pathway have their cells of origin intermixed mainly within VTA regions and these innervate the amygdala (Amg), olfactory tubercle (OT) and the lateral septum (LS), **(c)** mDA neurons that project their axons along the mesocortical pathway have their cells of origin intermixed between SNc and VTA subpopulations and innervate the prefrontal (PFC), entorhinal and cingulate cortices. mesocortical pathways in the rat. VTA, ventral tegmental area; SNc, substantia nigra pars compacta, SNr, substantia nigra pars reticulate; SNl, substantia nigra pars lateralis.

1.6.2 mDA axonal targets in the forebrain

Tracing studies have also subgrouped these mDA neurons according to their axonal projections along three distinct projection pathways that innervate distinct target areas in the forebrain, each correlating to specific functions.

The A9-SN mDA neurons project along a nigro-striatal pathway to target the caudate putamen of the dorsal striatum. The A10-VTA mDA neurons project via the mesocortical pathway to the cortex (cingulate, prefrontal and perirhinal cortices) and mesolimbic pathways to innervate various limbic structures including the amygdala, olfactory tubercle and the septum. In addition to these targets, the A10 VTA mDA neurons also target the nucleus accumbens of the ventral striatum (Bjorklund and Dunnett, 2007). The anatomical location of the mDA neurons and their projection pathways to forebrain target areas have been summarised in Figure 1-5. Single-cell tracing of long- range projections neurons have divided mDA neurons of the VTA that terminate their axons in the forebrain into four main subtypes (Aransay *et al*., 2015). These are the (i) mesocorticolimbic; that project to both neocortex and basal forebrain, (ii) mesocortical; projecting exclusively to the neocortex, (iii) mesostriatal; terminating only in the caudate putamen of the striatum and (iv) mesolimbic; that project their mDA axons to the NAc and basal forebrain including lateral septal regions (Aransay *et al*., 2015).

1.6.2.1 Lateral Septum as a target of mDA neurons

In the subcortical forebrain, the lateral septum (LS), also sometimes referred to as the lateral septal nucleus (LSN), is anatomically located dorso-caudally to the nucleus accumbens and dorso-rostrally to the decussation of the anterior commissure and the hypothalamus (Sheehan *et al*., 2004). In rodents, the LS is located directly between the lateral ventricles (Sheehan *et al*., 2004). Like the midbrain, the LS is also considered to be a very heterogenous structure in regards to its cellular organisation, neurochemical properties, dynamic range of efferents and afferent connections to several other brain regions.

1.6.2.1.1 LS Anatomical Organisation

Swanson and Cowan, (1979), Verney *et al*., (1987) and several others since have regionally divided the LS into its cytoarchitectonic subdivisions with their dorsal (LSd/ DLS/ LSNd), intermediate (LSi/ ILS/ LSNi) and ventral (LSv/ VLS/ LSNv) components (Swanson and Cowan, 1979; Verney *et al*., 1987; Sheehan *et al*., 2004). For the purpose of this work and simplicity, the LS components will be referred to in this work and resuming chapters as dorsal (LSd), intermediate (LSi) and ventral (LSv) as shown in Figure 1-6. Swanson and Cowan (1979) have detailed the definitions of these LS components clearly as follows:

LSd - *"The dorsal part of the lateral septal nucleus is triangular in outline when seen in frontal sections; it lies immediately ventral to the angle between the corpus callosum and the lateral ventricle… The dorsal part of the lateral septal nucleus, as we have defined it, corresponds to the nucleus dorsalis pars externa of Andy and Stephan (1964)."* (Swanson and Cowan, 1979)

LSi – *"The intermediate part of the lateral septal nucleus is the largest and most heterogeneous subdivision of the lateral septal nucleus. The neurons of the inter- mediate division become more widely scat- tered as one approaches the medial septal nu- cleus, from which they are separated by the fibers of Zukerkandl's bundle."* (Swanson and Cowan, 1979)

LSv – *"A distinct ventral part of the lateral septal nucleus has been recognized in a number of species… It is separated ventrolaterally from the bed nucleus of the stria terminalis by a cell-free zona limitans."* (Swanson and Cowan, 1979).

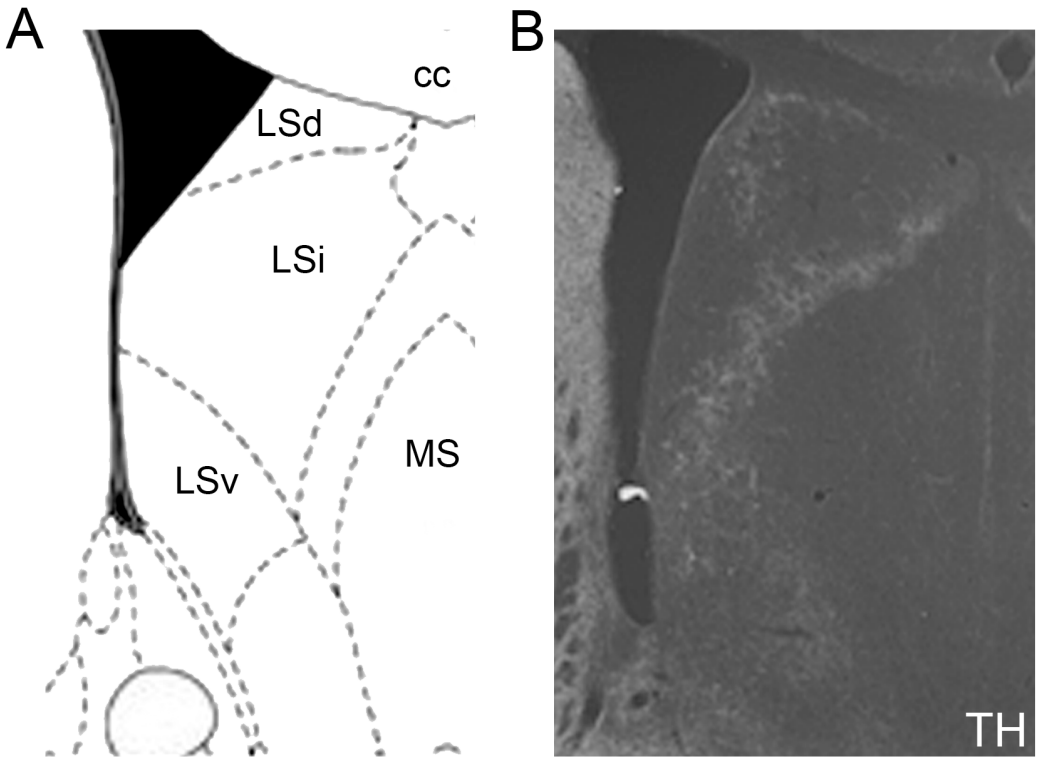

Figure 1-6 The subdivisions of the lateral septum and DA innervations in LS regions

A, The LS is subdivided into dorsal (LSd), intermediate (LSi) and ventral (LSv) regions. **B**, Antibody immunohistochemistry for TH reveals DA axons innervating both LSd and LSi components (white staining) in adult mouse brain. Cc, corpus callosom; LSd, dorsal lateral septum; LSi, intermediate lateral septum; LSv, ventral lateral septum; MS, medial septum; TH, tyrosine hydroxylase.

1.6.2.1.2 LS Neuronal Subtypes

The adult septum which includes the lateral (LS), medial (MS) and ventral (VS) septal nuclei is described as an 'onion-like' structure according to the distribution of neuronal marker expression in distinct subsets of septal neurons (Wei *et al*., 2012). Septal neurons are primarily GABAergic however a proportion of MS neurons are also cholinergic. Like mDA neurons, septal neurons are also extremely heterogenous; they are generated in development in an "outside-in" pattern giving

rise to five main subtypes (Wei *et al*., 2012). These consist of (i) parvalbumin PV+ GABAergic, (ii) choline acetyltransferase ChAT+ cholinergic, (iii) neuronal nitric oxide synthase nNOS+, (iv) calretinin CR+ GABAergic and (v) calbindin CB+ GABAergic neurons, which are distributed from ventral- medial to dorsal-lateral positions diagonal 'onion-like' strips along the septum (Wei *et al*., 2012). Of these neuronal subtypes, LS neurons however consist of GABAergic neurons. The LSi neurons express calbindin (CB-immunoreactive) and the LSv neurons express calretinin (CR-immunoreactive) as neuronal markers.

1.6.2.1.3 LS Connectivity

The LS sends efferent output to a wide range of brain regions and also receives abundant afferent glutametergic inputs from the hippocampus. Additionally, those brain regions associated with the regulation of emotional states including the amygdala, medial prefrontal cortex, bed nucleus of the stria terminalis and entorhinal cortex also send afferent projections to the LS. Brain areas that control motivational responses such as hypothalamic and midbrain regions also send afferents to integrate connections within the LS. Of the midbrain regions, the LS receives inputs from the locus cerulus, raphe nucleus, dorsal tegmentum (Cornwall *et al*., 1990) and VTA which send their adrenergic, serotonergic, cholinergic and dopaminergic (Gaspar *et al*., 1985) projections respectively.

1.6.2.1.3.1 DA axons terminating in LS

The regulation of autonomic, endocrine and behavioural responses to stressful environmental circumstances are primarily mediated by the involvement of mDA

axon projections to the LS and BNST (Sesack, 2002; Lindvall and Stenevi, 1978). The LS receives DA innervation from VTA, these make direct synaptic contact with perikarya and dendrites of septal neurons (Gaspar *et al*., 1985). These connections produce both excitatory (Assaf and Miller, 1977) and inhibitory postsynaptic responses. Several studies throughout the 1970s and 1980s have reported the identification of dopaminergic fibers by observations of DA/ TH immunoreactivity in more than one components of the LS in discrete axonal arrangements.

Two distinct types of DA axon fibers were observed within the LS as described by Lindvall in 1974. One DA fiber type observed in the LS was of spherical spindle-like shape that was regularly spaced with fine varicosities (Lindvall, 1975). These fibers had intervaricose segments that were difficult to visualise (Lindvall, 1975). In contrast, the second DA fiber type observed in the LS was clearly visible as smooth fibers without many varicosities (Lindvall, 1975). Furthermore, this DA axon type was often seen outlining unlabelled non-fluorescent neuronal cell bodies and proximal dendrites of the LS and formed pericellular arrangements (Lindvall, 1975). Ultrastructural analysis of DA fibers by immunocytochemistry using the peroxidase method and anti-DA antibodies in LS regions was also investigated by Onteniente *et al*., (1984). Onteniente *et al*., (1984) described the DA axons that innervate along the LSi region as "*packed in a band incurving from the border of the lateral ventricle to the medial part of the lateral septum"* (Onteniente *et al*., 1984). These DA axons were observed along the entire rostro-caudal extent of the LS (Onteniente *et al*., 1984; Lindvall, 1975).

According to Lindvall and Stenevi (1978), the fully developed adult LS contained DA axons along four longitudinal fields or strips along its rostro-caudal extent. One DA

strip in rostral LS, two main DA strips along the medial and lateral extent of the middle septum and one last DA strip in caudal LS. Describing the two main DA strips of the medial and lateral portions of the middle septum, Lindvall and Stenevi (1978) identify dopaminergic innervations to the lateral septal area observed in rat as one that originates from the medial part of the A10 mDA cell group of the VTA (Lindvall and Stenevi, 1978). Specifically, DA axons from the VTA form two distinct terminal patterns in the LS, one that is smooth forming pericellular arrangements around LS cells (Lindvall and Stenevi, 1978; Lindvall, 1975). This DA axon type is observed in both LSi and appears scattered in LSd regions (Lindvall, 1975). The second type of DA axons that form fine-varicose axons into a dense band sheet around the fornix are found within the LSi region, and also have their cells of origin in the VTA (Lindvall and Stenevi, 1978).

Despite these observations, detailed molecular and cellular characterisations of the VTA-specific mDA cells of origin that project their axons to the LS have not been thoroughly documented. Although several lesion studies (Lindvall, 1975), LS-injected horseradish peroxidase (HRP) retrograde labelling (Assaf and Miller, 1977; Lindvall *et al*., 1977; Carter and Fibiger, 1977; Fallon and Moore, 1978) and autoradiographic anterograde (Fallon and Moore, 1978) studies have shown the overall distribution of mDA cells in the VTA that project to the LS, detailed molecular characterisation of these septal-projecting VTA-specific mDA neurons, such as specific molecular markers, or factors important for the survival and correct axonal targeting to this region have not been investigated.

Although DA innervations have been visualised in the LS, one of the VTA nuclei implicated in direct projection to the LS is the PN of the VTA (Aransay *et al*., 2015).

All PN mDA neurons are mesolimbic in the sense that they send axons only to limbic forebrain targets (Aransay *et al*., 2015). Furthermore, whether mDA projections to the LS are exclusive or whether these axons project collaterals to additional target areas within the forebrain is not clear.

Single-cell axon tracing of long-range projection neurons of the VTA by transfection of the Sindbis-pal-eGFP vector in mice combined with axon labelling with iontophoretic microdeposits of biotinylated dextran amine (BDA) and reconstruction of individual axonal trees from serial sections, revealed two individual neurons of the PN in the VTA that projected axons exclusively to the LS (Aransay *et al*., 2015). However, one mDA neuron from the PN projecting to the LS, terminal arbor in the LS measuring 10.7mm, also displayed axonal branching to the NAc (Aransay *et al*., 2015). These findings support further heterogeneity of mDA neurons projecting to forebrain structures and the molecular mDA subset projecting to the LS still need to be investigated in detail. Furthermore, molecular markers need to be identified that can label functional mDA neurons projecting to LS regions.

1.6.2.1.4 LS Functions

The lateral septum with its connections to several other brain regions, is implicated in many behaviours. However, the specific activation of DA neurotransmission in LS and the behavioural functions of LS functioning in a dopamine context have been studied in a limited number of studies implicating this circuit in the modulation of the septo-hippocampal pathway (Robinson *et al*., 1979), learning (Yamamuro *et al*., 1995), sensory-related processing related to stress (Adams and Moghaddam, 2000) and drug addictive behaviours. Particularly, the actions of amphetamine (Renard *et*

al., 2014), cocaine (Sotomayor-Zarate *et al*., 2013; Reddy *et al*., 2016; Harasta *et al*., 2015) and alcohol (Jonsson *et al*., 2015) as drugs of abuse on DA elevation, release and uptake within the LS have been investigated to date.

Renard *et al*., (2014), investigated the withdrawal effects of chronic amphetamine (AMPH) on inducing changes in DA transmission within the LS. Male Sprague-Dawley rats were injected with AMPH over 14 days and then subjected to withdrawal periods ranging from 24 hours to 14 days. AMPH-treated rats displayed significantly reduced potassium (K+)-induced release of extracellular DA levels in the LS measured by *in vivo* microdialysis after 14 days of AMPH withdrawal compared to saline-treated controls (Renard *et al*., 2014). No changes were observed however in DA tissue content or TH expression levels although VMAT levels were significantly lower after 14 days of AMPH withdrawal of AMPH-treated rats compared to saline-treated controls. This study was the first to demonstrate changes in K+-induced DA release in the LS resulting directly from withdrawal after repeated AMPH administration (Renard *et al*., 2014). The results also provide a link in reduced DA releasability as a result of reduced DA vesicular uptake and implicate AMPH abstinence syndrome in the role of DA afferents within the LS.

Functional properties of DA innervations of the LS were also investigated by assessing action-potential mediated DA release in LS using microdialysis after subjecting male Sprague-Dawley rats to handling, which is a form of mild stress for these otherwise calm rat breed (Adams and Moghaddam, 2000). Once septal baseline DA levels were established, animals were handled in their cage and subjected to restraining of their movement in the cage for 20 minutes. During this

period, septal extracellular DA levels significantly increased to 140% of baseline DA in response to handling (Adams and Moghaddam, 2000). DA levels remained this high in the LS an hour after handling and only normalised to basal DA state 80 minutes after termination of handling (Adams and Moghaddam, 2000). After DA levels returned to basal pre-stress levels, local application of the sodium channel blocker, TTX, through the microdialysis probe produced significant decline in DA levels below detection demonstrating that previously measured DA was of neuronal origin (Adams and Moghaddam, 2000). This study implicates functions of DA innervation within the LS to sensory information processing of stress.

Glucagon-like peptide 1 receptor (GLP-1R) is expressed at peak mRNA levels in the LSd portion of the LS. The LSd is an anatomical site that has a crucial function in reward perception. Functional relevance of GLP-1R expression within the LSd is in driving behavioural responses to cocaine due to increased excitability of these cells in whole-cell patch-clamp recording as a result of genetic ablation of *Glp-1r* (Harasta *et al*., 2015).

Another study links GLP-1R-mediated regulation of the cocaine action with DA homeostasis within the LS (Reddy *et al*., 2016). DA terminals, visualised by DAT staining within the LSi, were found to be positioned adjacent to GLP-1R- expressing cells within the dorso-medial aspect of the LSi in both rostral and caudal LS sections of mApple BAC transgenic mice under the control of the GLP-1R promoter (Reddy *et al*., 2016). Furthermore, cocaine administration via the microdialysis probe increased septal extracellular DA levels by ~5 fold and systemic administration of a GLP-1R agonist Exendin-4 was able to prevent this rise in extracellular DA in reduce c-fos expression within the LS (Reddy *et al*., 2016). In addition to systemic, local

activation of GLP-1R, by co-perfusing the GLP-1R agonist Exendin-4 and cocaine via microdialysis probes, also blocked cocaine-induced elevation of extracellular DA levels. Hence septal neuropeptide GLP-1R signalling modulates cocaine's ability to elevate DA by promoting an increase in DAT expression within the LS thereby increasing DA uptake (Reddy *et al*., 2016).

Although these studies have provided with some insights into DA effects on the LS, the direct function of the DA afferents from the VTA to the LS have not been fully elucidated and the functional relevance of this brain pathway in terms of behaviour has not been clearly determined.

1.6.3 mDA neuronal functions & disease

The A9-SN mDA neurons function in regulating motor control and movement thus the specific loss and degeneration of these neurons, particularly of the SNpc, is the underlying pathological hallmark of PD. The resulting loss of SN mDA neurons results in clinical symptoms of PD related to motor impairment including gait disturbance, resting tremor, bradykinesia and rigidity.

The mesocortical and mesolimbic pathways include projections from the A10-VTA and A8-RRF to their relative targets, collectively function to regulate emotional behaviour, cognition, memory, learning and reward mechanisms (Tzschentke and Schmidt, 2000; Ang, 2006). Dysfunction of these pathways are implicated in depression (Dailly *et al*., 2004), psychotic symptoms in schizophrenia (Sesack and Carr, 2002), attention-deficit hyperactivity disorder (ADHD; Bjorklund and Dunnet, 2007; Lammel *et al*., 2008) and drug addiction (Kelley and Berridge, 2002; Wightman

and Robinson, 2002).

1.6.4 Electrophysiological phenotypes & circuitry/inputs

DA neurons also exhibit difference in the kind of information they integrate and their electrophysiological output firing patterns. SN mDA neurons that project along the mesostriatal pathway, exhibit a typical action potential (AP) waveform with a slow regular discharge pattern with prominent afterhyperpolarisations (AHPs) and sag components (Lammel *et al*., 2008). VTA mDA neurons that project along mesocortical pathways exhibit a broad AP waveform with a smaller amplitude and these APs have smaller maximal depolarization and repolarisation speeds (Lammel *et al*., 2008). The discharge pattern is fast and regular, without any AHPs and sag components (Lammel *et al*., 2008). This study highlighted the two distinctive functional phenotypes of the SN and VTA mDA neurons.

mDA neurons can also be further categorised into two distinct types according to their response to aversive stimuli. An aversive stimulus can be defined as any negative stimulus with undesirable consequences. SN-mDA neurons are mainly saliency coding, which are activated in response to aversive events. In contrast, VTA-mDA neurons are reward-value coding which are inhibited by aversive stimuli (Matsumoto and Hikosaka, 2007; Bromberg-Martin *et al*., 2010; Watabe-Uchida *et al*., 2012).

The whole brain mapping study of the direct monosynaptic inputs to the SN and VTA mDA areas conducted by Watabe-Uchida *et al*., (2012) using the rabies virus provided an insight into these differences in saliency and value coding of mDA neurons. This difference was attributed to them receiving different excitatory inputs; SN-mDA neurons from the subthalamic nucleus and the somatosensory/ motor

cortex in contrast to the VTA-mDA neurons from the lateral hypothalamus (Watabe-Uchida *et al*., 2012). Besides this, the study highlighted the diversity of areas from which information is integrated by synaptic input to mDA neurons of both the SN and VTA, including inputs from the septum to VTA (Watabe-Uchida *et al*., 2012).

1.6.5 mDA subsets

Specific recent studies have now suggested that mDA neurons within each sub-population also represent heterogenous and mixed subsets. Microarray based gene profiling of the A9-SN and A10-VTA specific subpopulations by laser capture microdissection (LCM) techniques, revealed a number of transcription factors that are preferentially expressed in either the SNc or the VTA including those only expressed in small subsets (Chung *et al*., 2005a). SNc-specific mDA subsets include Sox6 (Panman *et al*., 2014) amongst several others (Chung *et al*., 2005a). VTA-specific mDA subsets include orthodenticle homeobox 2 (Otx2; Di Salvio *et al*., 2010), Calbindin D28K (Liang *et al*., 1996a), aldehyde dehydrogenase family 1 (Aldh1a1/ Raldh1; Chung *et al*., 2005ab; Jacobs *et al*., 2007), Nolz1 (Panman *et al*., 2014) and neurogenic differentiation factor-6 (NeuroD6).

1.6.5.1 SNc-specific mDA subsets

1.6.5.1.1 Sox6

An SNc specific marker is the transcription factor Sox6, which was identified in a screen for mDA neuron-specific expressed genes (Panman *et al*., 2011). In the cortex, Sox6 is important for interneuron subtype diversity (Azim *et al*., 2009; Batista-Brito *et al*., 2009). Sox6 expression was investigated in the ventral midbrain by

immunohistochemistry at embryonic E18.5 and adult stages by Panman *et al*., 2014. At E18.5, Sox6 protein expression was localised in post-mitotic mDA neurons of the lateral SNc TH+ mDA subpopulation and corresponded to 95% of the total SNc mDA neurons that co-expressed TH and Sox6 and later in the adult Sox6+ accounted for 85% of the total SNc TH+ mDA subpopulation (Panman *et al*., 2014). Further characterisation of the Sox6+ mDA subset with additional molecular markers confirmed the status of Sox6 as an SNc-specific mDA subset marker since Sox6+ mDA neurons co-localised with SNc markers Girk2 and glycoDAT. Raldh1 is a marker for a proportion of both the ventral SNc and VTA mDA neurons. The Sox6 mDA subset localised in the SNc, also co-localised with the SNc compartment of Raldh1 expressing mDA subpopulation but not the VTA in both embryonic E18.5 and adult ventral midbrain. In support of Sox6 being an SNc- specific mDA subset and an SNc- specific molecular marker, Sox6+ mDA neurons do not co-express any VTA molecular markers including Otx2 and Calbindin, which are both absent from the Sox6 subpopulation in both embryonic and adult Sox6+ mDA subset. After molecular characterisation of the Sox6 population, the study identified the dorsal- lateral striatum as the specific projection target site of Sox6+ mDA neurons in the SNc (Panman *et al*., 2014). This was demonstrated by retrograde tracing experiments using fluorgold injected into the dorsal striatum, which retrograde labelled majority of the Sox6+ mDA neurons in the SNc in the adult brain (Panman *et al*., 2014).

During the early development of mDA neurons, at E11.5, Sox6 expression is confined to the medial portion of Corin+ Lmx1a+ mDA progenitors where they also express low weak levels of Otx2 but do not express Nolz1. This subset is referred to as the Sox6+/Otx2+weak/Nolz1- subpopulation of mDA progenitors already

established at E11.5 and later becomes the lateral SNc (Panman *et al*., 2014). Sox6 is then continued to be expressed in early post-mitotic immature Nurr1+/ TH- mDA neurons. Between E13.5 and E18.5, differentiating post-mitotic Sox6+ mDA neurons are found medially in the IZ of the mDA domain and these Sox6+ mDA cells first migrate ventrally and then extend laterally along the MZ and form the lateral SNc subpopulation of mature mDA neurons (Panman *et al*., 2014). Functionally, Sox6 may regulate the expression of axon guidance molecules required for correct innervation to the dorsal-lateral striatum and for the maintenance of SNc-like mDA properties since the loss of Sox6 causes a reduction in mDA axonal innervations within the doral lateral portion of the striatum in Sox6 mutants (Panman *et al*., 2014). Sox6 is also expressed in human neuromelanin- containing SNc mDA neurons and Sox6 levels are significantly diminished in patients with PD compared to control individuals as measured by densitometry of Sox6-expressing neuromelanin-containing mDA neurons (Panman *et al*., 2014). These findings implicate Sox6+ SNc neurons as a functional mDA subset and implicate Sox6 in the maintenance of SNc-like properties of mDA neurons and PD.

1.6.5.2 VTA-specific mDA subsets

1.6.5.2.1 Otx2

The important roles of Otx2 in early stages of mDA neural development are well known. Otx2 is expressed in mDA progenitors cells as early as embryonic day (E) 9 (Ang *et al*., 1994). Otx2 is required for determining the molecular code and specification of mDA progenitors by regulating the extent, identity and fate of the mDA progenitor domain in the ventral midbrain (Puelles *et al*., 2003; 2004) and mDA

neurogenesis (Vernay *et al*., 2005). For the early stage functions of Otx2 in mDA progenitor specification, see reviews (Smidt and Burbach *et al*., 2007; Ang, 2006).

1.6.5.2.1.1 VTA-specific expression of Otx2

In addition to its expression and function in progenitor specification, from E12.5, Otx2 is also expressed by a subset of post-mitotic embryonic TH+ mDA neurons that are fated to generate VTA-specific mature mDA neurons (Di Salvio *et al*., 2010a). At E15.5 and E18.5, Otx2+/TH+ double positive cells continue to increase along the rostral-caudal extent of the VTA, whilst there is no expression of Otx2 in the SN (Di Salvio *et al*., 2010a). In the adult, there is graded expression Otx2 in TH+ neurons along the dorso-ventral VTA. In the dorsal VTA, very low numbers of TH+ neurons (8%) co-localise with Otx2, whereas Otx2 expression is maintained in a high percentage of TH+ mDA neurons localized in the central (medial tier) (37%) and ventral (75%) VTA (Di Salvio *et al*., 2010a). An LCM approach was used by Chung *et al*., 2005, 2010 to isolate VTA and SN mDA neurons and showed Otx2 mRNA levels to be elevated by up to 6 fold in the VTA compared to the SN of the adult mouse (Chung *et al*., 2005; Chung *et al*., 2010) and human (Chung *et al*., 2010).

1.6.5.2.1.2 VTA-specific functions of Otx2

1.6.5.2.1.2.1 Subtype identity

Using a *Cre* mutant mouse model to study the function of Otx2 in adult mDA VTA neurons, Di Salvio *et al*., 2010b found that Otx2 functions to control molecular subtype identity of central VTA neurons by restricting the expression of Girk2, to the dorsal-lateral VTA neurons instead of central VTA neurons.

1.6.5.2.1.2.2 Projection targeting

To elucidate the role of Otx2 in regulating the axonal projection patterns of VTA mDA neurons, Chung *et al.,* 2010 validated potential candidate transcriptional targets from their previous microarray study (Chung *et al.,* 2005a). Overexpression, using lentivirus encoding Otx2, and endogenous reduction of Otx2, using short hairpin RNA (shRNA) directed against Otx2, in MN9D cells and/or ventral mesencephalic cultures, revealed that in the VTA, Otx2 is required for the upregulation of axon guidance molecules including Neuropilin 1 (Npn1), Neuropilin 2 (Npn2) and Slit2 (Chung *et al*., 2010).

Subsequent analysis of adult Otx2 conditional knockout (cKO) mice, that were previously reported to display a significant reduction in both VTA and SN mDA neurons due to the loss of Otx2 expression (Borgkvist *et al*., 2006), revealed a dramatic reduction in VTA mDA neurons in nearly all VTA-specific target areas including the PFC, NAc, OT, amygdale and the septum (LS and dorsal; Chung *et al*., 2010). Together, these results indicate the possible function of Otx2 in establishing correct projection targeting of VTA mDA neurons. Whether this is directly and independently of other transcription factors, and additional downstream targets, is still unclear.

1.6.5.2.1.2.3 Neuroprotection

Otx2 functions to modulate dopamine signaling by limiting the number of VTA TH+ mDA neurons with efficient reuptake of dopamine by restricting high expression levels of glycol-DAT to dorsal-lateral VTA Girk2+/TH+ neurons instead of central and

ventro-medial VTA mDA neurons (Di Salvio *et al*., 2010b) hence negatively regulating DAT expression. Since Otx2 negatively regulates DAT expression in VTA mDA neurons, Otx2 functions as a neuroprotective factor for VTA mDA neurons that express Otx2. This was analysed by assessing the sensitivity of VTA and SN-specific mDA subpopulations that express and do not express Otx2, respectively, to the neurotoxin MPTP. MPTP-treated Otx2 mutants displayed a significant reduction in GFP+/TH+ VTA neurons compared to control mice treated with MPTP, and showed a similar sensitivity to MPTP as wild-type SN mDA neurons. Since MPTP is a dopamine analog, it can be efficiently bound by glyco-DAT and so by negatively regulating DAT expression, Otx2 confers neuroprotection for the Otx2-expressing VTA mDA neurons. To confirm that the difference in increased susceptibility to MPTP-induced neurodegeneration of SN mDA neurons compared to that of the VTA, is largely due to the neuroprotective role of Otx2, ectopic expression of Otx2 in the SN showed an increase in the resistance of these SN mDA neurons upon MPTP treatment (Di Salvio *et al*., 2010b).

The neuroprotective function of Otx2 was confirmed by an independent study by Chung *et al*., 2010 in which Otx2 was overexpressed in ventral mesencephalic cultures using lentivirus to validate putative transcriptional targets. Otx2 overexpression revealed an upregulation of neuropeptides including vasoactive peptide (VIP) and adenylyl cyclase activating peptide (Adcyap; Chung *et al*., 2010). Treating ventral mesencephalic cultures with the MPP neurotoxin, after overexpression of Otx2 with lentivirus encoding Otx2, revealed a 25% increase in Otx2+/TH+ mDA neurons. Whereas reduction of Otx2 using shRNA, resulted in 25% decrease in Otx2+/TH+ mDA neurons. Since Otx2 regulates neuropeptides such as VIP and Adcyap, which are known to exhibit neuroprotective effects (Baranowska-

Bik *et al*., 2013; Chung *et al*., 2005), the mechanism by which Otx2 expression renders VTA mDA neurons less susceptible to neurodegeneration is by upregulating neurotrophic and neuroprotective target genes (Chung *et al*., 2010).

1.6.5.2.2 Calbindin D28K

Majority of Otx2+ mDA neurons within the central and ventral VTA co-express Calbindin D28K (CB, Di Salvio *et al*., 2010a). CB is expressed in VTA neurons that are more resistant to neurodegeneration and thus has crucial neuroprotective functions (Mattson *et al.,* 1991; German *et al*., 1992; Lavoie and Parent, 1991; Liang *et al*., 1996) by inhibiting apoptosis via the activation of the PI3-kinase-Akt signaling pathway (Sun *et al*., 2011). Further support for a pro-survival function and neuroprotective effects of CB are from the analysis of adult Pitx3-hypomorphic aphakia mice that have selective degeneration of SNc and dorsal VTA neurons that do not express CB (CB-negative mDA neurons; Luk *et al*., 2013).

1.6.5.2.3 Nolz1

Nolz1 is a zinc finger protein which is a member of the NET zinc finger protein family (Chang *et al*., 2013). It is important for neuron subtype diversification of striatal progenitor and post-mitotic differentiating neurons of the striatum (Chang *et al*., 2013).

In mature mDA neurons, Nolz1 is selectively expressed in VTA mDA neurons and not the SNc. Embryonically, Nolz1-expressing mDA neurons correspond to 18% of the total VTA mDA subpopulation at E18.5 and less than 10% of the VTA subpopulation in the adult ventral midbrain (Panman *et al*., 2014).

At E11.5, during the early development of mDA neurons at the progenitor stage, Nolz1 expression is in the laterally positioned mDA progenitors and these Nolz1+ mDA progenitors strongly co-express Otx2 yet are mutually exclusive to medially positioned Sox6-expressing mDA progenitors. Hence this lateral subpopulation is referred to as the Nolz1+/Otx2strong/Sox6- subpopulation of mDA progenitors, which later form a part of the medial VTA subpopulation providing evidence of an 'outside-in' pattern of mDA neuron development (Panman *et al*., 2014). The function of Nolz1 in VTA neurons still remains to be determined.

1.6.5.2.4 Aldh1a1

Retinaldehyde dehydrogenase (Aldh1a) also known as Raldh1, is an enzyme that catalyses the oxidation of retinaldehyde into retinoic acid (RA). Aldh1a1 is expressed in mDA progenitor cells and the expression of this protein becomes restricted to a proportion of mDA neurons in the adult (Chung *et al*., 2005b). In mature mDA neurons, Aldh1a1 is expressed in a subset of both ventral SNc and ventral VTA subpopulations and its expression is regulated by Pitx3 in these neurons (Jacobs *et al*., 2007; Chung *et al*., 2005b). In the adult VTA, Otx2+ mDA cells account for 75% of TH+ neurons in the ventral VTA region. Aldh1a1 is expressed in all ventral Otx2+, Calbindin+ mDA cells of the VTA (Di Silvio *et al*., 2010). Aldh1a1 synthesises RA, which is neuroprotective for mDA neurons as demonstrated by treatment of *Pitx3* homozygous mutant mice with RA during mDA development resulting in SNc recovery by partial rescue of Aldh1a1 expression (Jacobs *et al*., 2007). The early role of Aldh1a1 in mDA progenitors remains to be determined however in adult VTA mDA neurons, Aldh1a1 also mediates the alternative non-canonical synthesis of GABA, independent from glutamate decarboxylase (GAD), facilitating the co-release

of GABA and DA from these ventral VTA neurons (Kim *et al*., 2015). This GABA co-release is modulated by ethanol consumption at high binge-drinking alcohol concentrations. The functional relevance of diminished Aldh1a1 in this VTA subset leads to increased alcohol consumption implicating reward-based drug abuse and addictive behaviours (Kim *et al*., 2015).

It is clear that VTA-specific mDA subsets are determined by the expression of key neuroprotective pro-survival molecular markers in dorsal, central and ventral tiers of the VTA. These represent distinct functional mDA subsets projecting to specific forebrain targets with unique roles in determining mDA circuitry and subsequently the modulation of specific DA behaviours. Evidence provided here has shed light on the molecular and functional heterogeneity of mDA neurons in the VTA. Since VTA mDA neurons and their vast axonal targets in the forebrain are implicated in several rewarding and aversive behaviours such as addiction and stress/ social sensory information processing, this complexity of mDA neurons needs to be unravelled in more detail at a more molecular level.

1.7 bHLH Transcription Factors

The family of basic helix-loop-helix (bHLH) transcription factors are crucial proteins that regulate many developmental pathways and comprise of more than 130 related and evolutionary conserved proteins in humans (Skinner *et al*., 2010). The number of bHLH genes increases with increasing complexity of organisms due to genetic duplications and phylogenetic diversification (Skinner *et al*., 2010; summarised in table 1-1; See appendix 7.2 for a list of human bHLH proteins).

Organism	Name	Number of bHLH genes
Baker's yeast	Saccharomyces cerevisiae	8
Worm	Caenorhabditis elegans	39
Fly	Drosophila melanogaster	58
House mouse	Mus musculus	104
Humans	Homo sapiens	125

Table 1-1. Phylogenetic diversification. The number of bHLH genes increases with increasing complexity of organism.

Murre *et al*., defined HLH proteins as *"regulatory factors that when expressed in the appropriate cell type induce the expression of a number of genes, resulting in a tissue-specific phenotype."* (Murre *et al*., 1994).

1.7.1 Structure of bHLH proteins

The bHLH domain spans 60- 100 amino acids in length. A bHLH transcription factor protein can be structurally defined by its two primary domains; a basic region, which is required for its binding to DNA to a consensus DNA sequence (CANNTG) called the E-box sequence, and the helix-loop-helix domain, which is characterised by two α-helices separated by a loop (Bertrand *et al*., 2002). The HLH domain is required for dimerisation of the protein to form hetero- or homo-dimers (Lee, 1997).

1.7.2 Classification of bHLH proteins

The bHLH transcription factor proteins can be classified according to their spatial and temporal expression, specific properties, species of origin and phylogenetic homology (Murre *et al*., 1994). Classification by Murre *et al*., divides bHLH proteins into the following six broad classes I – VI (Murre *et al*., 1994).

Class I bHLH proteins are ubiquitously expressed and can form both hetero- and homodimers. These are mainly E-proteins including E12 (TCF3) and E2-2 (TCF4). These proteins generally act as transcriptional activators and are involved in the gene regulation of B cells, muscles and pancreatic tissues.

The expression of bHLH proteins belonging to class II is restricted to specific tissues where they are able to control the expression of downstream genes in a tissue-specific manner and regulate cell-intrinsic differentiation by forming heterodimers with other ubiquitously expressed class I bHLH proteins. Class II is the largest class of bHLH proteins and consist of those that regulate muscle differentiation include Myogenin, MyoD and Myf-5. Of those expressed in the brain, class II bHLH protein families include the neurogenins, oligs and the NeuroD family.

Class III bHLH proteins are involved in growth control and have important roles in cancer. Myc related proteins including c-Myc, L-Myc (Mycl1) and N-Myc (bHLHe37) belong to this class.

Class IV consists of a very small group of bHLH factors that interact with Myc, including Mad and Max proteins.

HLH proteins that represent class V are those that do not contain a basic region therefore lack their DNA binding domain and include the include Emc and Id proteins. They form non-functional heterodimers acting as competitive antagonists of other bHLH factors.

Class VI proteins are characterised by the presence of a proline amino acid residue in their basic region and have high homology to the Drosophila ‘Hairy and Enhancer of split’ protein for example, the negative regulator Hes5 which is the main effector in Delta-Notch signalling.

The Neurod1 and Neurod6 bHLH transcription factors, which are the focus of this work, belong to the large class II of bHLH proteins (Bertrand *et al*., 2002). According to this classification, they are able to heterodimerise with other class I and class V bHLH proteins. Importantly, both Neurod1 and Neurod6 are mainly expressed throughout the CNS.

1.7.3 Neuronal bHLH transcription factor proteins in development

Several bHLH transcription factors are expressed throughout the CNS and play important roles during neuronal development. They are broadly divided according to whether they act early- or late- during neuronal development as (i) determination factors or (ii) differentiation factors.

Genes of determination factors are expressed early in embryogenesis and function in cell fate determination by initiating either glial or neuronal differentiation processes. They are expressed by multipotent progenitor and precursor cells in mitotic ventricular zones (VZ) of various brain regions. Since these factors are initiators of neuronal differentiation they are often termes as “proneural factors”. Examples of proneural determination bHLH factors are the Neurogenins (Ma *et al*., 1996) and Mash1 (Tomita *et al*., 2000).

In contrast, differentiation factors are not expressed in progenitor cells and are absent from mitotic VZ regions of brain. They are expressed slightly later in embryogenesis in already determined cell fates and committed neurons and therefore are termed as “neuronal bHLH proteins”. Examples of neuronal bHLH

factors include those belonging to the NeuroD family, Neurod1, Neurod2 and Neurod6.

Neuronal bHLH proteins, which include early determination factors and late-acting differentiation factors, can act as survival factors for the neurons in which they are expressed. In support of this, genetic inactivation studies of determination factors Ngn2/Mash1 for example, reveal a loss of neural progenitor cells (Andersson *et al*., 2006a; Kele *et al*., 2006). Alternatively, inactivation of late-onset differentiation factors such as Neurod1/6 reveal a loss of terminally differentiating granule neurons of the hippocampus due to increased cell death (Schwab *et al*., 2000). In this thesis, inactivation of Neurod1 and Neurod6 will provide further evidence to support the survival properties of differentiation factors.

1.8 NeuroD family of bHLH transcription factors

The NeuroD subfamily of bHLH transcription factors include Neurod1 (Lee *et al*., 1995), Neurod2 (Pleasure *et al*., 2000), Neurod4 (Tomita *et al*., 2000) and Neurod6 (Schwab *et al*., 2000) named accordingly due to amino acid sequence similarity of the bHLH domain. In addition to this, the sequence homology between these NeuroD family members extends a further 40 amino acids beyond just the bHLH domain. This 40 amino acid sequence includes an atypical leucine repeat interspersed with proline residues (Lee, 1997). Importantly, all members of this gene family are “single-exon-encoded-genes” (Lee, 1997).

According to Bertrand *et al*., (2002), the NeuroD family of bHLH proteins have characteristics of differentiation factors. Firstly, they are expressed in immature or mature neurons rather than in progenitor cells. Secondly, they possess the ability to promote neuronal differentiation when ectopically expressed (Lee *et al*., 1995).

1.8.1 Neurod1

Neuronal differentiation factor 1 (NEUROD1) also known as BETA2, BHF-1, MODY6, NeuroD and bHLHa3 was initially cloned by Lee *et al*., (1995) from a yeast two-hybrid protein interaction assay that investigated Neurod1's ability to heterodimerise with a protein in *Drosophila* called Daughterless. The hamster homolog of Neurod1 was also discovered by Naya *et al*., (1997). The Neurod gene maps to human chromosomal location of 2q32 and in mouse chromosome 2 (Tamimi *et al*., 1996). Generally, Neurod1 is highly expressed in regions where differentiating neurons reside and is expressed in many organs both within the PNS and CNS. In the PNS, Neurod1 is expressed transiently in the cranial and dorsal root ganglia (DRG) and otic vesicle. In the CNS, Neurod1 is expressed from around E11.5 and maintained into adulthood within the olfactory epithelium, olfactory bulb, retina, pineal gland, hippocampus, cerebellum and the cortex (Lee, 1997). Neurod1 expression is thought to be biphasic in the sense that one wave of expression is transient during neurogenesis, whilst a second wave of expression is in mature differentiated neurons (Lee, 1997).

1.8.1.1 Neurod1 Functions

1.8.1.1.1 Cell fate specification & Neuronal differentiation

Neurod1 has essential roles in cell fate specification and differentiation of subtypes of pancreatic islet cells (Chao *et al*., 2007). Analysis of *Neurod1*-null mice revealed a significant reduction in alpha and beta cells of the pancreas after E17.5 (Chao *et al*., 2007). Neurod1 is also required for the differentiation of granule cells in the hippocampus and cerebellum (Miyata *et al*., 1999).

1.8.1.1.2 Cell survival

Neurod1 is cell intrinsically required for the survival of mature and adult-born neurons of the hippocampus and olfactory bulb (Gao *et al*., 2009). Apart from the CNS, Neurod1 is essential for normal development of mammalian vestibular and auditory systems in the PNS (Liu *et al*., 2000; Jahan *et al*., 2010). Neurod1-null mutant mice exhibited behavioural impairments including reduced responses to sound, hyperactivity, circling behaviour and head tilting (Liu *et al*., 2000). Further analysis of these mutants revealed inner ear defects particularly a significant reduction in the number of sensory neurons in the cochlear-vestibular ganglion (CVG) between E13 and E18.5 (Liu *et al*., 2000). At E13, cell quantification revealed a 30% and 40% reduction in cochlear and vestibular neurons, respectively. However, loss of CVG neurons became more severe during embryonic development and by E18.5 95% of cochlear and 85% of vestibular neurons were already lost in *Neurod1* mutants compared to controls. Few remaining neurons survived until adulthood. The loss of CVG neurons in these mutants were further analysed to determine whether the loss is due to less proliferation of CVG to begin with or whether CVG neurons are lost

due to apoptotic cell death. Analysis of proliferative activity prior to neuron loss revealed by BrdU immunohistochemistry in these mutants showed proliferation was unaffected and the loss of CVG neurons in these mutants were as a result of delayed and defective delamination and increased apoptotic neuronal death (Liu *et al*., 2000) suggesting Neurod1 plays a major role in the maintenance and survival of post-mitotic differentiating and terminally-differentiated neurons. Further studies on the mouse ear have also demonstrated Neurod1's requirement in the survival and formation of axonal connections (Jahan *et al*., 2010). Conditional deletion of floxed Neurod1 was generated specifically for the ear using TgPax2-Cre mice and these were also compared with systemic *Neurod1*-null mice. These conditional Neurod1 floxed mice survive after birth and in postnatal mice, Neurod1 is required for the survival of most spiral and vestibular neurons of the mouse ear and for axon projections of surviving cochlear afferents. In the absence of Neurod1, the axon connections of the surviving cells are disorganised (Jahan *et al*., 2010). Hence in addition to the fundamental role of Neurod1 in subtype-specific neuronal survival, Neurod1 is also crucial for the proper development of axon connectivity within the mammalian ear (Jahan *et al*., 2010).

Neurod1 also controls tumour cell survival of neuroendocrine lung carcinomas and promotes metastasis via the regulation of downstream target genes; receptor tyrosine kinase B (TrkB), which is the receptor for brain-derived neurotrophic factor (BDNF) and neural cell adhesion molecule (NCAM) (Osborne *et al*., 2013).

Neurod1 cKO mice under the control of cone- rod homeobox (Crx) allowed for the selective expression of Neurod1 specifically in the cone and rod photoreceptor cells of the retina (Ochocinska *et al*., 2012). Analysis of these mutants revealed the

requirement of Neurod1 also in the survival of retinal photoreceptors (Ochocinska *et al*., 2012).

1.8.2 Neurod2

The *Neurod2* gene was mapped to chromosomal location 17q12 in human and chromosome 11 in mouse (Tamimi *et al*., 1997). Neurod2, also known as NDRF and bHLHa1, was first cloned and characterised by McCormick *et al*., (1996). Northern blot analysis detected Neurod2 expression from E11.5. Neurod2 expression levels peak shortly after birth and then declines to weaker expression levels (like Neurod6, see later; Schwab *et al*., 1998). Neurod2 expression is maintained in adulthood in regions of the CNS; cortical plate of the cortex, hippocampus, dendate gyrus and cerebellum (McCormick *et al*., 1996; Schwab *et al*., 1998). Since Neurod2 has a later onset of expression during embryogenesis and was found to activate the promoter of a more downstream gene, the GAP-43 gene (McCormick *et al*., 1996; like Neurod6, see later), Neurod2 is considered a downstream regulator of neurogenesis.

1.8.2.1 Neurod2 Functions

Neurod2 induces the neural differentiation of cortical projection neurons, mouse embryonic stem cells and is required for proper development of the amygdala (Bayam *et al*., 2015; Sugimoto *et al*., 2009; Messmer *et al*., 2012; Noda *et al*., 2006; Lin *et al*., 2005). In addition to neural differentiation, Neurod2 is essential for proper synaptic development of thalamocortical axons and patterning of the barrel cortex (Ince-Dunn *et al*., 2006). *Neurod2*-null mice, in which the entire *Neurod2* coding region is replaced by β-galactosidase (Olson *et al*., 2001; Lin *et al*., 2004) were used

to observe Neurod2 expression and patterning defects. *Neurod2*-null mice exhibited a disorganisation and severe disruption of the cortical barrel field as revealed by antibody staining for mitochondrial cytochrome oxidase in thalamo-cortical axon terminals (Ince-Dunn *et al*., 2006). Lipophilic DiI labelling revealed that in the absence of Neurod2, these thalamo-cortical axons fail to segregate (Ince-Dunn *et al*., 2006). Neurod2 is also required for the correct development and maturation of thalamo-cortical synaptic connections (Ince-Dunn *et al*., 2006). *Neurod2*-null mice lack both the lateral and basolateral amygdala nuclei and mice heterozygous for Neurod2 have reduced number of neurons in the amygdala (Lin *et al*., 2005). Behavioural analysis of *Neurod2* heterozygous mice in fear learning tests revealed severe defects in emotional learning by alterations in key molecular regulations of emotional learning including the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and γ-Aminobutyric acid (GABA) receptors as a result of Neurod2 absence (Lin *et al*., 2005). Hence the dosage of *Neurod2* is critical in regulation of genes involved in amygdala fear processing and emotional behaviour (Lin *et al*., 2005).

1.8.3 Neurod4

Neurod4 also known as MATH-3 was initially cloned as Beta3 by Peyton *et al*., (1996) using partial degenerate primers binding other class II bHLH genes and the hamster insulin tumour cDNA library. Human Neurod4 was also independently cloned by Horikawa *et al*., (2000) by RT-PCR of adult human pancreatic islet mRNA. The *Neurod4* gene maps to chromosomal location 12q13.2 in humans (Horikawa *et al*., 2000) and chromosome 10 in the mouse (Tsuda *et al*., 1998). *Neurod4* expression was detected robustly in the chicken dorsal root ganglia, spinal cord, retina,

diencephalon and metencephalon however *Neurod4* transcripts were absent from the telencephalon/ cortex and a layer lining the ventricular zone (VZ) (Roztocil et el., 1997). Since, class II bHLH proteins are not usually expressed in the VZ, Neurod4-expressing cells however are located at the interface between the proliferative and non-proliferative zones of the VZ where they have just left the cell cycle and have begun differentiating yet have not begun the migration process. Hence, Neurod4 was characterised to define the early and transient stage when a cell begins the differentiation process (Roztocil *et al*., 1997).

1.8.4 Neurod6

Neurod6 alternatively known as Nex1, MATH2 and ATOH2, belongs to the NeuroD subfamily of bHLH transcription factors (Guo *et al.*, 2002) and has structural homology to the product of the Drosophila proneural gene *atonal*. Neurod6 was independently cloned in rat by Shimizu *et al*., (1995) and Bartholoma and Nave (1994) using degenerate primer and RT-PCR from rat cDNA. The *Neurod6* gene of chromosomal location 7p15-p14 consists the two non-coding exons 1a and 1b, the single coding exon 2 and two intronic regions (Uittenbogaard *et al.*, 2007). *Neurod6* is transcriptionally regulated via two promoter regions P1 and P2 that are under the control of CRE and C/EBP regulatory elements respectively (Uittenbogaard *et al.*, 2007). The NeuroD6 protein has a molecular size of 38.6 kDa and is 337 amino acids in length (Shimizu *et al*., 1995), with a glutametergic-rich N-terminal domain, 2 transactivation domains (TAD1 and TAD2), surrounding the nuclear localization (NL) and the bHLH domains (Uittenbogaard *et al*., 2007). The human *Neurod6* gene was also cloned from a human foetal brain cDNA library (Guo *et al*., 2002) which is highly homologous to mouse *Neurod6* with a 98% protein sequence similarity differing in

only four amino acids. Furthermore, both the human and mouse bHLH domains have 100% sequence similarity (Guo *et al*., 2002).

1.8.4.1 Neurod6 expression in the brain

Whole- mount *in situ* hybridisation on mouse embryos detected *Neurod6* expression in the CNS from E11.5 in brain vesicles and the spinal cord (Shimizu *et al*., 1995). Later between E15.5 and E18.5, *Neurod6* expression was detected more prominently in the cortical plate and mantle layer throughout the developing CNS however it is not ever expressed in the ventricular zone where progenitor cells are dividing. Therefore, Neurod6 expression is restricted to post-mitotic differentiating committed neurons. In the adult, Neurod6 expression is maintained in a number of regions throughout the brain detected in cortex, olfactory bulb, dentate gyrus, hippocampus and the cerebellum (Shimizu *et al*., 1995; Schwab *et al*., 1998; Schwab *et al*., 2000). Particularly, high levels of *Neurod6* RNA are detected in the cortex however in other neural tissue regions the RNA levels are relatively lower. Furthermore, Neurod6 expression is specific to the nervous system since it is not detected in non-neural tissues (Shimizu *et al*., 1995).

Since Neurod6 is expressed in several brain regions, changes in Neurod6 levels have been implicated in a number of clinical disorders including schizophrenia (Perez-Santiago *et al*., 2012), attention-deficit/ hyperactivity disorder (Ribases *et al*., 2009), obsessive compulsive disorder (Mattheisen *et al*., 2015), heteropia (Castro *et al*., 2002), epilepsy (Dixit *et al*., 2016; Elliott *et al*., 2001) and alzheimer's disease (Barral *et al*., 2015; Fowler *et al*., 2015; Li *et al*., 2015; Satoh *et al*., 2014).

In the mesencephalon, *Neurod6* expression is detected at E11.5 (Lee, 1997) however the precise regions within the mesencephalon or in which neurons was not precisely documented in previous literature. In the microarray based gene profiling study by Chung *et al*., (2005) mentioned previously, a 5-fold enrichment of *Neurod6* expression was found in VTA-specific mDA neurons of the ventral midbrain compared to the SNc. However, the temporal and spatial distribution of Neurod6 in mDA neurons of the VTA has not been investigated. In this thesis, I will unravel the temporal and spatial expression patterns of Neurod6 specifically in mDA neurons.

1.8.4.2 Role of Neurod6 in neurons

1.8.4.2.1 Cell Fate & Subtype Identity

In the CNS, another model of cellular diversity giving rise to functionally distinct cell types is the vertebrate retina. Retinal cells belong to a neuronal cell class that includes distinct cell types that have similar molecular, morphological and physiological neuronal properties that underlie shared function (Masland, 2004; Cherry *et al*., 2011). Neurod6 also has key roles specifically in regulating cell fate and defining subtype identity of retinal cells (Cherry *et al*., 2011; Kay *et al*., 2011). In the adult retina, Neurod6 is normally expressed by a subset of non-GABAergic, non-glycinergic amacrine cells. Misexpression of Neurod6 by electroporation at P0 induces amacrine cell fate more strongly and this alters neurite targeting of some of these amacrine cells (Kay *et al*., 2011; Cherry *et al*., 2011).

1.8.4.2.2 Axonal Outgrowth & Synaptic Plasticity

In the CNS, apart from cell developmental processes such as neuronal differentiation, transcription factors belonging to the bHLH family have also been implicated in late-stage functions including neurite outgrowth, target selection and synaptogenesis. Neurod6 functions to promote axonal outgrowth and synaptic plasticity via the regulation of downstream genes involved in synaptic remodelling and neurite extension. These include the growth associated protein 43 (GAP-43; Uittenbogaard *et al*., 2003) and the plasticity related gene 1 (*Prg1*; Yamada *et al*., 2008) are both direct targets of Neurod6. Neurod6 is capable of mediating neurite outgrowth in the absence of nerve growth factor by activating GAP-43 (Uittenbogaard *et al*., 2003).

1.8.4.2.3 Differentiation & Neuronal Survival

Neurod6 has key functions the maintenance of the differentiated state of neurons and in neuronal survival (Uitenbogaard and Chiaramello, 2005; Uitenbogaard *et al*., 2010a). The precise mechanism by which Neurod6 promotes neuronal survival, similar to nerve growth factor (NGF), includes both the maintenance of expression of G1 phase cyclin-dependent kinase inhibitors and activation of key anti-apoptotic regulators (Uittenbogaard and Chiaramello, 2005). Key components of the Neurod6-mediated transcriptional network that regulates neuronal survival are the $p27^{kip1}$, $p21^{WAP/cip1}$ and $p16^{INK4a}$ CDK inhibitor proteins belonging to the CIP/KIP and INK4 families (Uittenbogaard and Chiaramello, 2005).

Neurod6 also has a neuroprotective role in tolerance to mitochondrial oxidative stress by enhancing biogenesis (Baxter *et al*., 2012; Uittenbogaard *et al*., 2010b) and

in cytoskeletal organization (Kathleen Baxter *et al*., 2009). This highlights its cellular functions that may be important for the regulation of basic neuronal machinery and properties. Ultimately, Neurod6 is widely expressed throughout the CNS and has important functions in neuronal survival in cooperation with other members of the NeuroD family (Schwab *et al*., 2000; Bormuth *et al*., 2013). However, the precise expression patterns and role of Neurod6 in the midbrain dopamine region has not been investigated. This presents a gap in our knowledge of the unknown expression profiles and roles for NeuroD family of bHLH transcription factors in mDA neurons, which are a highly heterogeneous and diverse population of neurons, in the developing and adult brain. This thesis will address this gap in knowledge and provide further insights into the molecular heterogeneity of mDA neurons in relation to their axon target sites in the forebrain as well as the important roles for Neurod6 and Neurod1 in promoting their survival.

1.9 Research Questions & Aims of this Thesis

(i) Identification and molecular characterisation a novel mDA subset marked by the expression of Neurod6. This will be the main focus of Chapter 3.

Objectives of this chapter will be firstly to characterise the temporal and spatial expression pattern of Neurod6 within the midbrain, documenting this for the first time. Secondly, to assess this mDA subset in relation to known and established molecular markers of mDA subpopulations and to other yet understudied less well documented molecular markers.

(ii) To determine the role of Neurod6 in mDA neurons. This will be addressed in Chapter 4.

Using a loss-of-function approach in *Neurod6* mutants, the objectives will be to characterise any cellular mDA phenotypes in the VTA that may provide evidence for the function of Neurod6 in mDA neurons.

(iii) To identify the mDA projection target site(s) within the forebrain of this Neurod6-marked mDA neuronal subset. This will also be addressed in Chapter 4.

The objective here will be to firstly characterise any mDA axonal phenotypes in relevant forebrain regions of *Neurod6* mutants. Secondly, to demonstrate

projections to specific forebrain targets of Neurod6-marked mDA neurons using fluorogold retrograde tracing methods.

(iv) To determine roles for additional members of the NeuroD family of proteins in a Neurod6+ mDA subset. This will be presented in Chapter 5.

Objectives of this chapter will be to firstly, elucidate expression of other NeuroD family members since these have been shown to have overlapping expression profiles in the brain. Secondly, since NeuroD members exhibit redundant roles in the hippocampus and cortex, generation of double mutants and assessing any cellular changes as a result of loss of more than one factor will be addressed.

Chapter 2. Materials & Methods

2.1 Generation and genotyping of mutant embryos and animals

We first generated *Neurod6*$^{Cre/+}$;*R26R*$^{YFP/YFP}$ mice (referred to henceforth as *Neurod6* control mice) by sequential breedings of *Neurod6*$^{Cre/+}$ animals (Goebbels *et al*., 2006) with *R26R*$^{YFP/YFP}$ reporter mice. We intercrossed control *Neurod6*$^{Cre/+}$;*R26R*$^{YFP/YFP}$ mice to generate *Neurod6*$^{Cre/Cre}$;*R26R*$^{YFP/YFP}$ single homozygous mutant embryos and mice. For double mutant studies, *Neurod1*$^{LacZ/+}$;*Neurod6*$^{Cre/+}$;*R26R*$^{YFP/YFP}$ mice were generated by breeding *Neurod1*$^{LacZ/+}$ mice (Miyata *et al*., 1999) with *Neurod6*$^{Cre/Cre}$;*R26R*$^{YFP/YFP}$ animals. These animals were then intercrossed to generate double homozygous mutant embryos and mice carrying different numbers of *Neurod1* and *Neurod6* mutant alleles. *Neurod6* heterozygous and homozygous allelic deletions were determined by PCR genotyping for the *Cre* transgene and *Neurod6*-wild-type (WT) primer sequences. *Neurod1* heterozygous and homozygous allelic deletions were determined by detection of the *LacZ* transgene and *Neurod1*-WT primer sequences. Table 2-1 shows the primer sequences used for genotyping of these animals. All animals used were from mixed background. At all times, animals were handled according to the Society of Neuroscience policy on the use of animals in Neuroscience research, as well as the European Communities Council Directive.

PCR	Forward primer sequence	Reverse primer sequence
Neurod6-WT	5'-GAGTCCTGGAATCAGTCTTTTTC-3'	5'-AGAATGTGGAGTAGGGTGAC-3'
Neurod6-Cre	CRE A CCGCATAACCAGTGAAACAG	Goebbels et al., 2006
Rosa26R-WT	5'-GCACTTGCTCTCCCAAAGTC-3'	5'-CTTTAAGCCTGCCCA GAAGA-3'
YFP	5'-GGGCGTACTTGGCATATGAT-3'	5'-GCGAAGAGTTTGTCC TCAACC-3'
Neurod1-WT LacZ	Miyata et al., 1999	Miyata et al., 1999

Table 2-1 List of primer sequences for genotyping

2.2 Tissue Preparation and Sectioning

For *in situ* hybridisation and immunohistochemistry experiments, 2 months old adult animals were deeply anesthetised with ketamine-xylazine (10mg/mL and 1mg/ML, respectively) and were transcardially perfused with 100mM phosphate buffered saline (PBS, PH 7.4) followed by 4% (wt/vol) formaldehyde in PBS, pH 7.4. Embryonic, postnatal and adult brains were removed, immersion-fixed in fixative 4% (wt/vol) formaldehyde in 100mM phosphate buffer, pH 7.4 overnight at 4°C and subsequently cryoprotected in 30% (wt/vol) sucrose-PBS. Tissue samples were embedded in optimum cutting temperature (OCT) compound (VWR International, Poole, UK), and sectioned on a cryostat (CM3050S; Leica, Nussloch, Germany) as 20 μm sections on Superfrost Plus microscope slides (25 x 75 x 1,0 mm; Thermo

Scientific) for embryonic brains and 35 – 50 μm free-floating sections for postnatal and adult brains.

For RNAscope experiments, adult animals were deeply anesthetised with ketamine-xylazine (10mg/mL and 1mg/ML, respectively) and were transcardially perfused with 10% neutral buffered formalin (NBF; Sigma-Aldrich). Brains were then removed and immersion-fixed in 10% NBF overnight at room temperature and then transferred into 70% ethanol for storage. Brains were subsequently embedded in paraffin wax and processed into 4 μm sections collected onto Superfrost Plus microscope slides (25 x 75 x 1,0 mm; Thermo Scientific) using the Leica RM2255 microtome.

2.3 *In Situ* Hybridisation

Section *in situ* hybridisations were performed as previously described (Vernay *et al*., 2005) or using the RNAscope® 2.0 HD Brown Chromogenic Reagent Kit according to the manufacturer's instructions (Advanced Cell Diagnostics, Hayward, CA). The following mouse antisense RNA probes have been used: *Neurod6* (Brohl *et al*., 2008), *Neurod1* (Lee *et al*., 1995), *TH* (Grima *et al*., 1985) and *Grp* (Wada *et al*., 1990). For RNAscope experiments, target probes for *Neurod6* and *Grp* were designed by Advanced Cell Diagnostics. For each probe, a minimum of three control and three mutant brains were analysed at embryonic and adult stages.

On day 1, sections were thawed at room temperature for 30 minutes. Slides were then washed three times in 1X diethylpyrocarbonate (DEPC)-treated PBS for 5 minutes. Slides were then transferred into 200ml of acetylation solution (2.66ml

Trietahnolamine (TEA), 0.32ml concentrated hydrochloric acid (HCl), 0.5ml acetic anhydride) for 10 minutes shaking at room temperature (RT). Slides were washed again in 1X DEPC-treated PBS for 5 minutes each, 3 times. Slides were then transferred into a humidified chamber containing Whattman filter paper soaked with 50% formamide, 5X SSC solution. Sections were then incubated in hybridisation buffer for 2 hours at RT making sure sections do not dry. Relevant probes were diluted in hybridisation buffer at 0.1g/ml (1:200 dilution, with a total of 200μl per slide). Once diluted, probes were denatured for 10 minutes at 95°C, vortexed and placed on ice for 5 minutes. Slides were covered with 200μl of diluted probes per slide and covered with glass coverslips to ensure even coverage of sections with probe. Slides were subsequently placed in a humidified chamber (containing filter paper soaked with 50% formamide, 5X SSC solution) overnight at 70°C in a water bath.

On day 2, slides were washed in 5X SSC solution at RT for 5 minutes until coverslips had fallen away from slides revealing sections. Sections were subsequently washed in 0.2X SSC twice for 30 minutes each at 70°C temperature in a water bath. After this, sections were washed again in 0.2X SSC solution for another 5 minutes this time at room temperature on the shaker. Slides were then transferred into Buffer 1 (0.1M Tris-HCl pH 7.5, 0.15M NaCl) solution for 5 minutes at room temperature. Sections on each slide were then covered in 1ml of blocking solution per slide for 2 hours at room temperature. Anti-digoxygenin antibody mix was made up to a 1:1500 dilution in buffer 1; 10% heat inactivated goat serum. After blocking, sections were incubated with 200μl of anti-digoxygenin antibody mix per slide, covered with parafilm and placed in a humidified chamber overnight at 4°C.

On day 3 of the *in situ* hybridisation experiments, parafilm was removed and slides were washed in buffer 1 solution 3 times, 5 minutes each at room temperature. Slides were rinsed in 1X NTMT twice for 10 minutes at room temperature. Staining solution made up fresh (3.5μl NBT, 3.5μl BCIP in 1X NTMT, 5% PVA solution per ml). 1ml of staining solution was placed evenly on each slide within a humidified chamber. Once covered in staining solution, slides were covered and left in humidified chamber in the dark at 37°C. Probe intensity signals were left to develop, ranging from few hours to day(s) depending on probe. If continuing staining the next day, sections were washed in 1X NTMT for 3 times for 10 minutes at room temperature and kept in the at 4°C overnight. If staining was completed, sections were washed first in 1X PBS to stop any further staining and the 3 times in water. Sections were left to dry and mounted with aquatex if not counter-stained with TH immunostaining. However, sections that required further immunostaining with TH antibody, were not dried but washed thoroughly in 1XPBS and incubated in appropriate primary antibody (see immunohistochemistry).

Solution (total volume)	Reagents used to make solution (ml)	Storage temperature
Pre-hybridisation buffer (50ml)	25ml deionised formamide 12.5ml 20X SSC (DEPC) 1.25ml tRNA (10mg/ml) 2.5ml 100X Denhardt's 2.5ml 10mg/ml herring sperm 6.25ml DEPC H_2O	-20°C
Buffer 1 (1L)	100ml 1M Tris-HCl pH 7.5 37.5ml 4M NaCl Distilled H_2O to 1L	RT
5X SSC (1L)	250ml 20X SSC 750ml H_2O Milli-Q®	RT
0.2X SSC (1L)	10ml 20X SSC 990ml H_2O Milli-Q®	RT / 70°C during incubation
Blocking solution (100ml)	10% NGS in 100ml Buffer 1 solution	4°C
NTMT 2X (500ml)	25ml 4M NaCl 100ml 1M Tris HCl pH 9.5 50ml 1M $MgCl_2$ 10ml 10% Tween-20 315ml H_2O Milli-Q®	RT/ 4°C if keeping slides overnight in this solution
50% formamide, 5X SSC (200ml)	100ml formamide 50ml 20X SSC 50ml H_2O Milli-Q®	RT
10% PVA (200ml)	20g PVA powder 200ml H_2O Milli-Q®	RT

Table 2-2 Solutions used for In Situ Hybridisation experiments and their volumes made per batch experiment

ml, millilitres; L, litre, SSC, saline- sodium citrate buffer; DEPC, diethylpyrocarbonate; mg, milligrams; pH, HCl, hydrochloric acid; NGS, normal goat serum; NaCl, sodium chloride; PVA, g, grams; RT, room temperature.

2.4 Immunohistochemistry

For immunohistochemistry, 20 μm frozen cryosections were thawed at RT for 30 minutes. Frozen cryosections or free-floating sections were washed 3 x 5minutes in 1X PBS and subsequently incubated in 1% (wt/vol) bovine serum albumin (BSA) in 100mM PBS blocking solution for 1 hour at RT. Sections were then incubated overnight at 4°C with the appropriate primary antibodies diluted in 1% BSA in 100mM PBS. On the second day, sections were washed thoroughly with 1X PBS and subsequently incubated for 1 hour at RT with appropriate secondary antibodies conjugated with a fluorochrome (Molecular Probes) diluted in 1% BSA in 100mM PBS. For nuclear staining, sections were incubated with 4',6- Diamidino-2-Phenylindole, Dihydrochloride (DAPI; 1:10,000). Sections were then washed extensively overnight in 100mM PBS and mounted in Vectashield H-1000 (Vector Laboratories,Burlingame, CA).

The following primary antibodies were used: sheep anti-GFP (1:1000; Bio-RAD/ AbD Serotec), rabbit anti-GFP (1:1000; Invitrogen), rabbit anti-TH (1:1000; Pelfreez), mouse anti-TH (1:500; Immunostar), sheep anti-TH (1:1000; Millipore); rabbit anti-OTX2 (1:500, Baas *et al*., 2000); goat anti-OTX2 (1:500, R&D Systems); mouse anti-CALBINDIN1 (1:1000, Swant); rabbit anti-ALDH1A1 (1:200, Abcam); rabbit anti-NOLZ1 (1:200, Biorbyt); mouse anti-NEUROD1 (1:500, Abcam); rabbit anti-NEUROD2 (1:1000, Abcam) and rabbit anti-TOM20 (1:1000; Santa Cruz Biotechnology).

The following secondary antibodies were used: Alexa Fluor 594 donkey anti-rabbit (1:300; Molecular Probes), Alexa Fluor 488 donkey anti-sheep (1:200; Molecular

Probes), FITC donkey anti-sheep (1:200; Jackson ImmunoResearch Laboratories, Inc.), Alexa Fluor 647 donkey anti-mouse (1:200; Molecular Probes), Cy5 donkey anti-mouse (1:200; Jackson ImmunoResearch Laboratories, Inc.), Cy5 donkey anti-sheep (1:200; Jackson ImmunoResearch Laboratories, Inc.).

2.5 Cell Counting & Imaging

For each brain, 50uM free-floating coronal cryosections were collected from the caudal to the rostral midbrain for immunohistochemistry with TH and GFP primary antibodies. Free-floating sections were subsequently mounted onto slides from caudal to the rostral midbrain and imaged using the Olympus Virtual Slide Microscope VS120-L100-W and ZEISS Apotome.2 Optical sectioning using structured illumination imaging systems. YFP+/TH+ double-positive cells were counted for one hemisphere of all midbrain sections to determine both the (i) total cell number and (ii) spatial distribution in controls and mutants at E18.5, P3, P7, P14 and adult stage. For each stage, three control and three mutant brains were analysed. Image J and Fiji (National Institutes of Health, NIH) software was used for cell quantification.

2.6 Statistical Analysis

Statistical analysis was performed only on cell counts of sections from the central mDA region. All sections through the central mDA region (bregma from -3.28 to -3.80 mm) contained 3 VTA nuclei, the PN, PBP and the posterior portion of the IF, SNc as well as two adjacent nuclei, the interpeduncular nucleus (IPN) and the red nucleus (RMC) (see schematic in Fig. 4-1I). Sections were matched according to the

presence of these landmarks from the caudal to the rostral extent. Besides these anatomical features, the most rostral section of the central mDA region also has the emergence of the fasciculus retroflexus. For cell count comparisons between two groups, statistical significance was assessed using the unpaired Student's t tests to determine differences in both the distribution and total number of TH+/YFP+ cells of the VTA in *Neurod6* controls and *Neurod6* mutants. When comparing cell number differences between more than two groups (*Neurod6* control, *Neurod6* mutant and *Neurod1;Neurod6* double mutants), statistical significance was assessed by One-way ANOVA (GraphPad Prism) with Tukey's post-test comparing the means of each group to the mean of every other group.

2.7 Assay for cell apoptosis

To detect cell apoptosis, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labelling (TUNEL) assays were performed using the TACS® 2 TdT DAB In Situ Apoptosis Detection Kit (Trevigen, R&D Systems; 4810-30-K). 10μM cryostat sections were immerse hydrated, fixed and immobilised in 1X PBS for 10 minutes, then covered with 50-200μl of Cytonin™ for 30 minutes. Samples were then washed twice in deionized water for 2 minutes each, then immersed in Quenching Solution (Methanol; 30% hydrogen peroxide) for 5 minutes. Samples were washed in 1X PBS for 1 minute, then immersed in 1X TdT Labelling Buffer for 5 minutes. Samples were covered with 50-200μl of Labeling Reaction Mix (TdT dNTP Mix; TdT Enzyme; 50X Cation Stock; 1X TdT Labeling Buffer) and incubated for 60 minutes at 37°C in a humidified chamber. Samples were then immersed in 1X TdT Stop Buffer for 5 minutes and washed twice in deionized water for 5 minutes each.

Samples were then covered with 50-100µl of Streptavidin-horse raddish peroxidase (Strep-HRP) solution and incubated for 10 minutes at 37°C in a humidified chamber to avoid evaporation. Samples were then washed twice in 1X PBS for 2 minutes each followed by immersion in DAB solution for up to 7-10 minutes. Samples were then washed twice again in deionized water for 2 minutes each. Once TUNEL was completed, sections were then analysed for TH expression by immunohistochemistry.

2.8 Densitometry

For the quantification of fluorescent intensity, images were acquired in structured illumination mode to achieve optical sectioning. Signal intensity of mitochondrial TOM-20 levels in YFP+/TH+ and YFP-/TH+ cells were quantified by Fiji image processing package. Regions of interest (ROIs) were accurately drawn around individual TH+ (YFP+ and YFP-) cells and readings of fluorescence intensity were only measured from the TOM-20 channel. Only mDA neurons showing the nucleus in the optic sections were included in the analysis (over 200 neurons were analysed per cell type per genotype, from at least 5 pictures per group), and background fluorescence values were subtracted from all the readings.

2.9 Retrograde fluorogold axonal labeling

The retrograde tracer Fluorogold (Millipore) was injected into the LS of *Neurod6*$^{Cre/+}$;*R26R*$^{YFP/YFP}$ control and *Neurod6*$^{Cre/Cre}$;*R26R*$^{YFP/YFP}$ mutant pups at postnatal day 10 (P10). Mice were anesthetized using isoflurane, and 1µl of Fluorogold (2% in 0.9% sodium chloride) and 2% BDA (Molecular Probes) was injected in both hemispheres. We adapted the stereotaxic coordinates of the atlas of

Developing Mouse Brain (Paxinos and Watson, 2007) and the glass microsyringe was placed in the brain with the tip at 0,3 mm anterior to Bregma, 0,2 mm lateral of the midline and 1,8 mm from the surface of the brain. Mice were sacrificed 3 days after injection for further analysis. Location of the injection sites were confirmed by staining the section with streptavidin- conjugated to Alexa Fluor 568 that binds to BDA.

Chapter 3. *Neurod6* is a novel marker for a subset of VTA mDA neurons

3.1 Introduction

mDA neurons are mostly found in the SNc and VTA and regulate multiple brain functions, including voluntary movement, working memory, emotion and cognition (Bjorklund and Dunnett, 2007). These neurons project to the forebrain and were initially thought of as a homogenous group of neurons based on their common use of dopamine as a neurotransmitter for intercellular communication. However, it is now becoming clear that mDA neurons are heterogenous in regard to their target and afferent projections (Roeper, 2013; Beier *et al*., 2015; Menegas *et al*., 2015), firing patterns (Roeper, 2013) and gene expression profiles (Poulin *et al*., 2014), all of which impact on their functional properties. mDA neurons projecting to striatal spiny projection neurons in the nucleus accumbens medial shell use glutamate as a co-transmitter (Hnasko *et al*., 2010; Stuber *et al*., 2010), whereas those projecting to the dorsal striatum use GABA (Tritsch *et al*., 2012). Furthermore, intact-brain analyses using a combination of whole brain imaging, optogenetics, viral tracing and fiber photometry, has revealed that different subsets of SNc neurons contribute to different nigrostriatal circuits carrying different information streams (Lerner *et al*., 2015).

Despite the emerging evidence for functionally distinct subsets of mDA neurons, we still know little of the molecular underpinnings of this functional diversity. Within the two classical subpopulations, the A9-SNc and A10-VTA, further complexity and new

molecular subsets can now be uncovered. It is necessary that these be identified and characterised in order to shed light on the heterogeneity of mDA neurons, unravel the roles of genes in individual mDA subsets and to understand differential vulnerability of mDA neurons in disease and dysfunction. Specific studies have investigated the heterogeneity of mDA neurons suggesting that mDA neurons within each sub-population also contain heterogenous and mixed subsets. Recent transcriptome analyses of VTA and SNc mDA neurons have provided lists of genes differentially expressed between these two anatomically separable groups of neurons (Grimm *et al*., 2004; Chung *et al*., 2005). Microarray based gene profiling of the A9-SN and A10-VTA specific subpopulations was conducted by Chung *et al*., 2005, revealing a number of differentially expressed genes between these two mDA subpopulations. mDA neurons specifically from the SNc and VTA were isolated by rapid TH-immunostaining followed by laser capture microdissection (LCM) of SNc and VTA neurons of adult mice. This was followed by microarray analsysis of differentially expressed genes and validation by RT-PCR. Their analysis revealed a number of transcription factors that are preferentially expressed in the VTA (Chung *et al*., 2005). These include orthodenticle homeobox 2 (Otx2; Di Salvio *et al*., 2010), Calbindin D28K (Liang *et al*., 1996) aldehyde dehydrogenase family 1 (Aldh1a1/ Raldh1; Chung *et al*., 2005; Jacobs *et al*., 2007). One of these differentially expressed genes is the basic helix-loop-helix transcription factor NEUROD6, alternatively known as NEX1, MATH2 and ATOH2. NEUROD6 belongs to the NEUROD subfamily of bHLH transcription factors. In the midbrain, there is preferential expression by nearly a 5-fold increase of *Neurod6* within the VTA compared to the SNc according to the microarray analysis of mDA subpopulations

(Chung *et al.,* 2005). However, the expression and role of NEUROD6 within the midbrain is unknown and not yet identified.

Neuropeptides, lipoproteins, growth factors and G-protein coupled receptors were also seen to be upregulated in the VTA compared to the SNc according to the LCM-microarray analysis conducted by Green *et al*., 2005 and Chung *et al*., 2005. These included gastrin releasing peptide (Grp), lipoprotein lipase (Lpl), insulin growth factor binding protein 4 (Igfbp4) and G-protein 83 (Gpr83; Chung *et al*., 2005). The expression patterns and roles of these molecules in VTA mDA neurons is still unknown.

In this chapter, I elucidate the expression of *Neurod6* within the VTA, document molecular characterisation of a *Neurod6*-marked mDA subset using gene expression analysis in Neurod6 control mice (Goebbels *et al*., 2006) according to known and unknown VTA markers.

3.2 Results

3.2.1 Neurod6 is expressed in a subset of post-mitotic VTA mDA neurons

I first examined the expression of *Neurod6* in the mouse midbrain by RNA *in situ* hybridisation from embryonic days 12.5 (E12.5) onwards. *Neurod6* expression was not detected at E12.5 (data not shown) but transcripts were found localised in the ventral regions of the VTA at E15.5 (Fig. 3-1A,A') and E17.5 (3-1B,B'). To determine whether *Neurod6* is expressed in mDA neurons, immunohistochemistry for TH protein expression was conducted following detection of *Neurod6* transcripts. *Neurod6* transcripts were co-localised with TH protein in the VTA at embryonic stages (brown staining in Fig. 3-1A,A',B,B') and its expression was maintained in a subset of ventral VTA TH+ neurons in adult mice (Fig. 3-1C-D'). In contrast, *Neurod6* was not detected in TH+ neurons of the SNc at any stage (Fig. 3-1C-D' and data not shown). Altogether, these results demonstrate that *Neurod6* is specifically expressed in a small subset of mDA neurons in the VTA from E15.5. to adulthood.

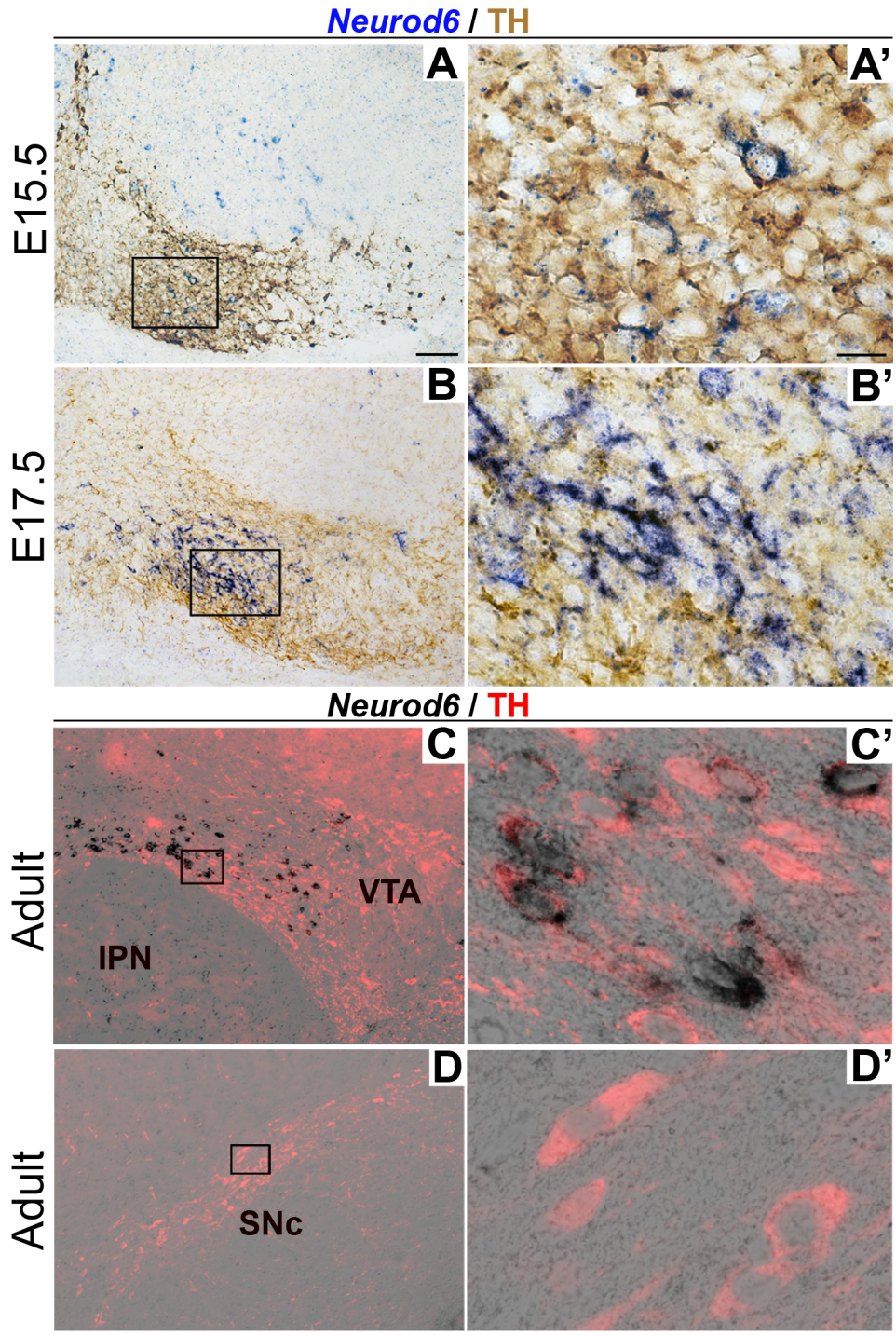

Figure 3-1 Selective expression of *Neurod6* in mDA neurons in the VTA

A-D, In situ hybridisation for *Neurod6* combined with immunohistochemistry for TH showing that Neurod6 expression is restricted to TH+ mDA neurons located in ventral regions of the VTA from E15.5 to adult stage (***A-C***). In contrast, Neurod6 is not expressed in TH+ mDA neurons in the SNc (***D***). ***A'-D'***, Higher magnification of boxed regions in corresponding panels A-D. Scale bars: (A) 200µm, (A') 10µm.

3.2.2 YFP faithfully mimics endogenous *Neurod6* expression in *Neurod6* control mice

To facilitate co-localisation studies between *Neurod6* and other genes expressed in mDA neurons in the VTA, and since a NEUROD6-specific antibody is not available, I used a mouse line where the coding region of *Neurod6* has been replaced by the *Cre* recombinase gene (Goebbels *et al*., 2006). I permanently labelled the cells in which the *Neurod6* promoter has been active by breeding these $Neurod6^{Cre}$ mice to $R26R^{YFP}$ reporter mice that conditionally express YFP in a Cre-dependent manner. I first confirmed that the CRE recombinase protein is co-expressed with YFP in VTA TH+ mDA neurons of $Neurod6^{Cre/+};R26R^{YFP/YFP}$ mice at P3 (Figure 3-2A) . I then showed that YFP expression is similar to endogenous *Neurod6* expression in the VTA of heterozygous $Neurod6^{Cre/+};R26R^{YFP/YFP}$ adult mice by conducting multiplex *in situ* hybridisation using RNAscope® followed by GFP antibody labelling. Indeed, all YFP+ neurons expressed *Neurod6* in the adult VTA (Figure 3-2B). Furthermore, since there is no working available antibody for NEUROD6, I used a pan-NeuroD antibody that detects all members of the NEUROD family of proteins, and found that all YFP+TH+ cells were pan-NEUROD+ in $Neurod6^{Cre/+};R26R^{YFP/YFP}$ adult control mice (Figure 3-2C). Altogether, these results establish that YFP expression in $Neurod6^{Cre/+};R26R^{YFP/YFP}$ mice faithfully reflects endogenous *Neurod6* expression, and that expression of *Neurod6* is maintained in neurons once it has been initiated. I therefore used YFP expression in these mice as a marker of *Neurod6*+ mDA neurons in subsequent experiments.

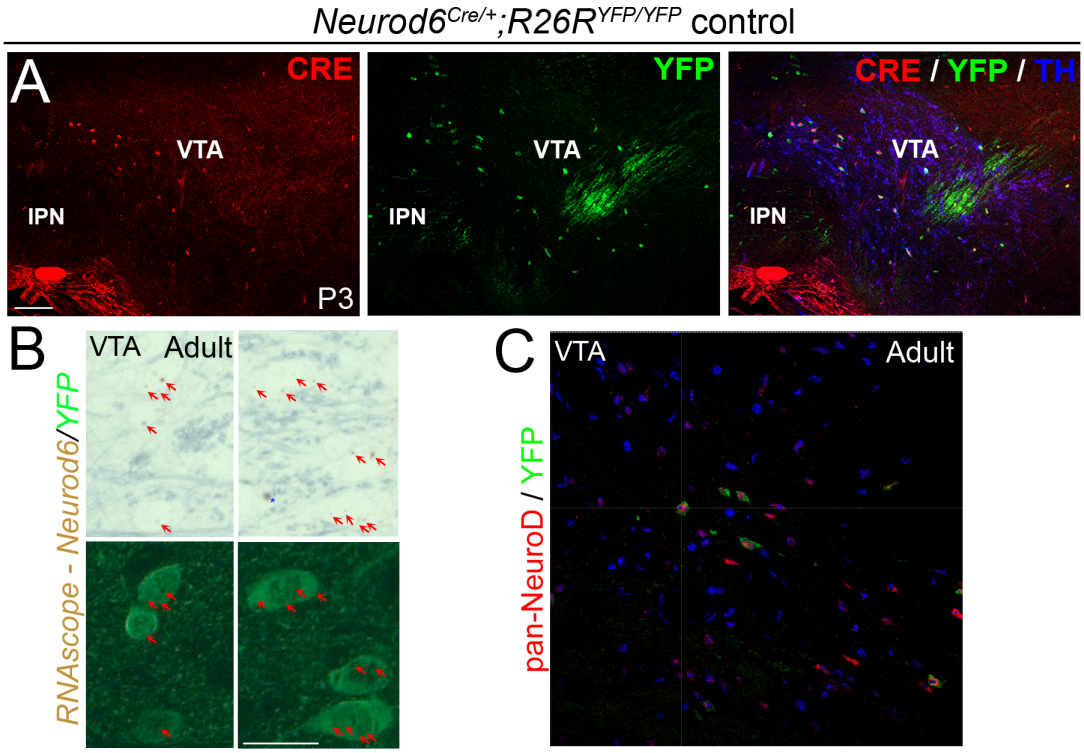

Figure 3-2 YFP+ cells express endogenous *Neurod6* in *Neurod6*$^{Cre/+}$;*R26R*$^{YFP/YFP}$ controls

A, Triple antibody labelling showing that CRE is co-expressed with YFP in TH+ mDA neurons in the VTA of *Neurod6*$^{Cre/+}$;*R26R*$^{YFP/YFP}$ pups at P3. ***B,*** In situ hybridization of *Neurod6* combined with immunohistochemistry for YFP showing that all YFP+ cells express *Neurod6* transcripts (brown dots shown by red arrows) in adult *Neurod6*$^{Cre/+}$ mice (blue asterix* is dust). ***C,*** Triple antibody labelling using a pan-NEUROD antibody that recognizes all members of the NeuroD family of bHLH transcription factors with YFP in TH+ mDA neurons in the VTA of *Neurod6*$^{Cre/+}$;*R26R*$^{YFP/YFP}$ adult mice shows that all YFP+ cells are pan-NEUROD+. Scale bars: (A) 100µm, (B) 20µm.

3.2.3 Neurod6 identifies a subset of OTX2+, ALDH1A1+, CALBINDIN1+ mDA neurons in the VTA

Earlier studies have shown that expression of the homeodomain-containing transcription factor OTX2 in the midbrain is restricted to mDA neurons in the VTA (Di Salvio *et al*., 2010b; Di Salvio *et al*., 2010a). Moreover, mDA neurons in the VTA can be further subdivided into dorsal, central and ventral layers based on co-expression of OTX2 with other markers including CALBINDIN1 and ALDH1A1 (Di Salvio *et al*., 2010a). I therefore performed triple antibody labelling experiments for TH, YFP and these three proteins individually, to compare their expression patterns with that of *Neurod6* in mDA neurons. The results show that all *Neurod6*+ cells express OTX2 (Figure 3-3A), CALBINDIN1 (Figure 3-3B) and ALDH1A1 (Figure 3-3C) indicating the existence of a group of VTA neurons co-expressing all four genes.

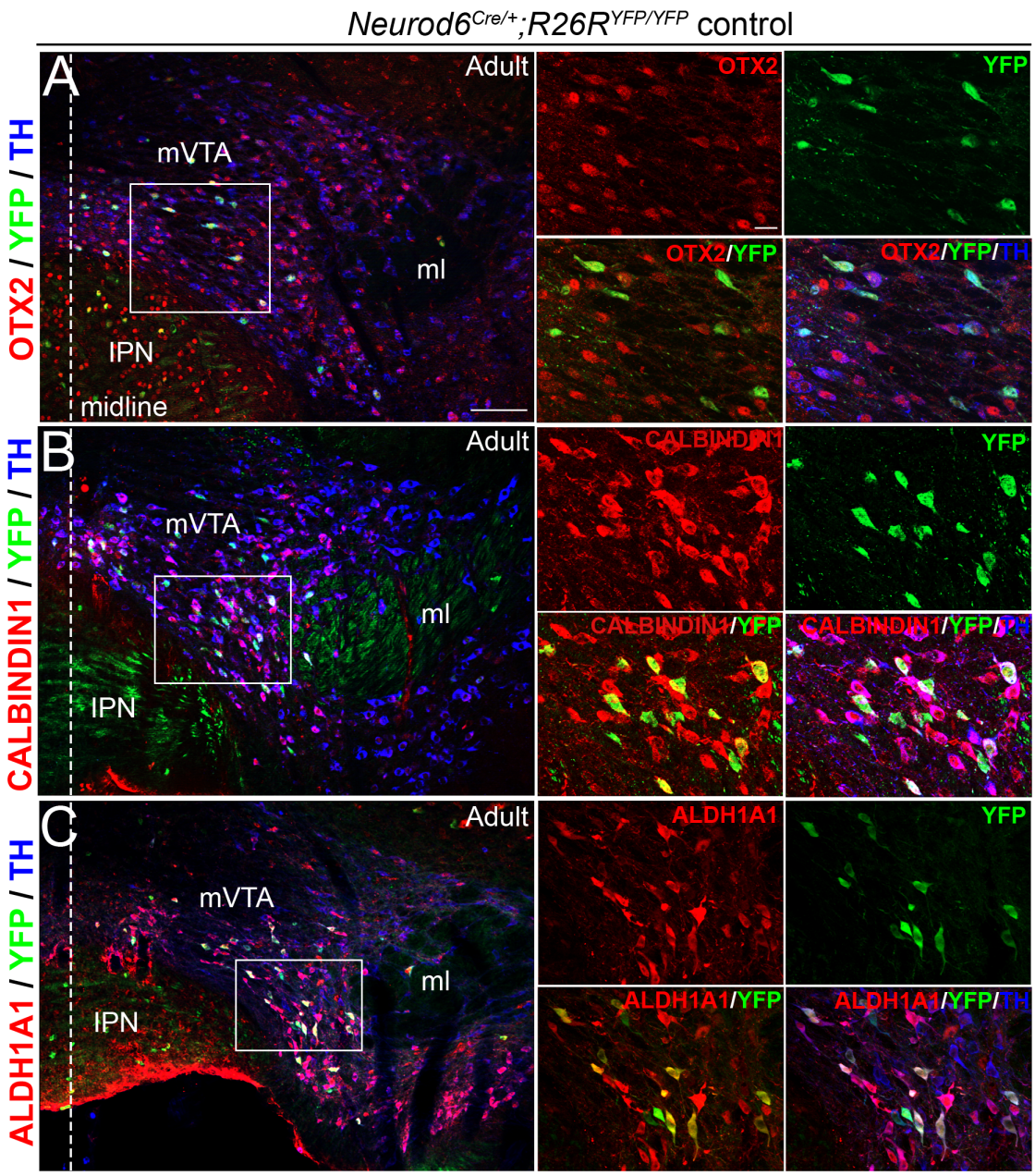

Figure 3-3 Identification of a novel subset of mDA neurons in the VTA that expresses *Neurod6*, OTX2, CALBINDIN1, ALDH1A1

A, B, C, Triple antibody labelling showing that all YFP+TH+ mDA neurons express OTX2 (***A***), CALBINDIN1 (***B***) and ALDH1A1 (***C***) in the VTA of adult *Neurod6*$^{Cre/+}$;*R26R*$^{YFP/YFP}$ mice. Higher magnification of boxed regions in corresponding panels. Scale bars: 100μm and high magnification panels 20μm.

3.2.4 Neurod6+ mDA neurons also express the VTA markers NOLZ1 and *Grp*

I also compared the expression of YFP and two other VTA-enriched genes, *Nolz1* (Panman *et al*., 2014) and *Grp* [GRP; Paul Allen Brain Atlas, (Chung *et al*., 2005)]. Triple antibody labelling experiments for TH, YFP and NOLZ1 proteins revealed that YFP+TH+ mDA neurons co-express NOLZ1 in *Neurod6*$^{Cre/+}$*;R26R*$^{YFP/YFP}$ adult controls (Figure 3-4A-D). NOLZ1 was also broadly expressed in other VTA neurons that were YFP-/TH+ (Figure 3-4A-D). *Grp* has been reported to be enriched in the VTA (Chung *et al*., 2005) however *Grp* mRNA cellular localisation in mDA neurons has not been observed. *In situ* hybridisation experiments for *Grp* followed by immunofluorescence antibody labelling of TH over the same sections, detected *Grp* mRNA transcripts in a subset of the ventral VTA of *Neurod6*$^{Cre/+}$*;R26R*$^{YFP/YFP}$ controls (Figure 3-4E). These *Grp* transcripts were localised specifically in TH+ mDA neurons of the ventral VTA (Figure 3-4E). RNAscope experiments using a *Grp* probe followed by antibody staining for GFP in *Neurod6*$^{Cre/+}$*;R26R*$^{YFP/YFP}$ adult mice revealed that *Grp* was expressed in all *Neurod6*$^{+}$ mDA neurons as well as in some surrounding YFP^{-} cells (Figure 2B). In summary, our expression studies have identified a novel subset of VTA mDA neurons that co-express *Neurod6*, OTX2, CALBINDIN1, ALDH1A1, NOLZ1 and *Grp*.

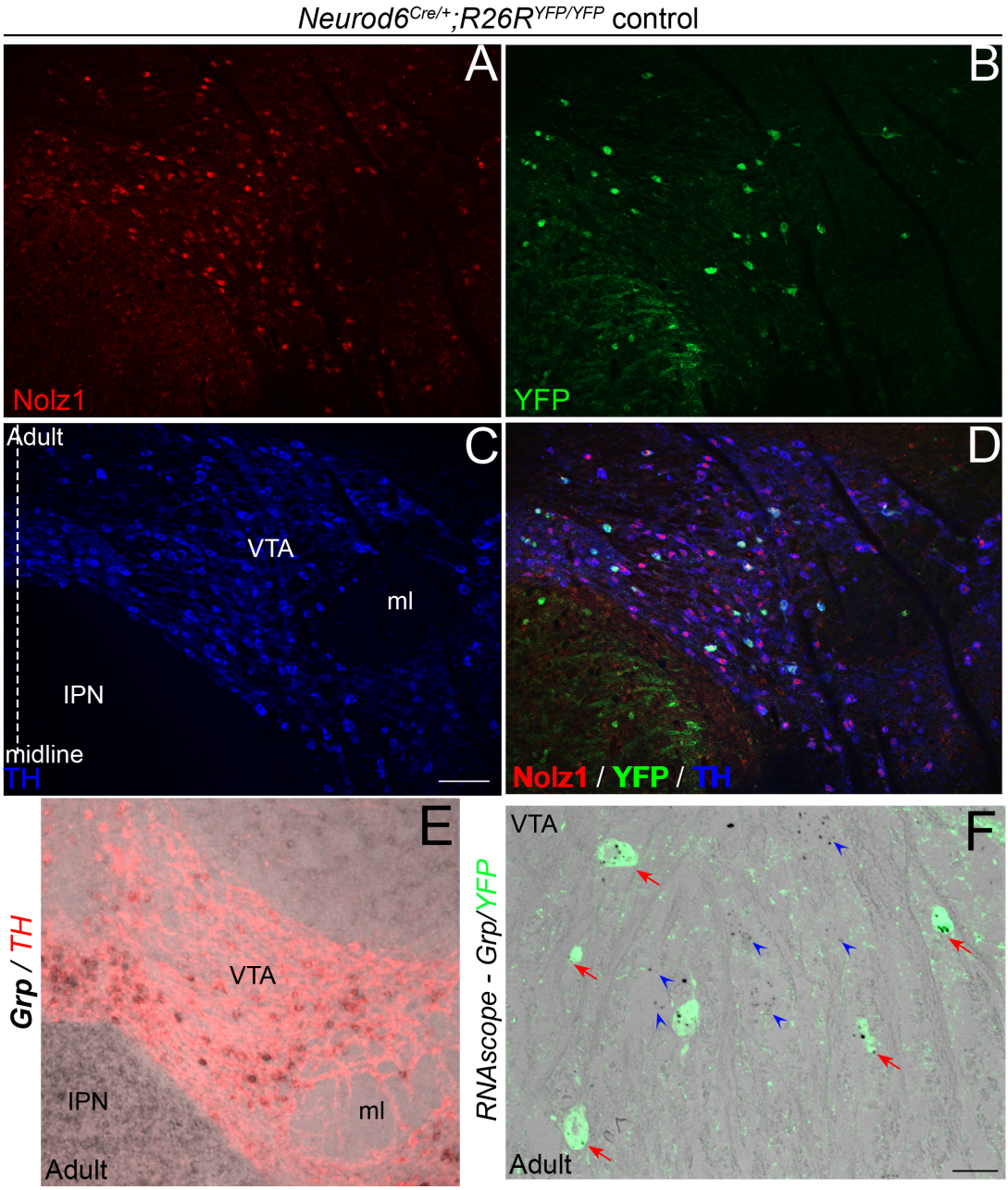

Figure 3-4 *Neurod6*+ mDA neurons of the VTA also co-express NOLZ1 and *Grp*

A-D, Triple antibody labelling showing that all YFP+TH+ mDA neurons express NOLZ1 (**A**) in the VTA of adult *Neurod6*$^{Cre/+}$;*R26R*$^{YFP/YFP}$ mice (***A-D***). (**E**) *In situ* hybridisation for *Grp* combined with immunohistochemistry for TH showing that *Grp* is expressed in TH+ mDA neurons located in ventral regions of the VTA in adult *Neurod6*$^{Cre/+}$;*R26R*$^{YFP/YFP}$ mice (***F***) *In situ* hybridisation (RNAscope) of *Grp* combined with immunohistochemistry for YFP showing that *Grp* transcripts are detected in both YFP+ (red arrows) and YFP- (blue arrowheads) cells in the VTA of adult *Neurod6*$^{Cre/+}$ mice. Scale bars: (A-E) 100µm, (F) 20µm.

3.3 Discussion

This chapter reveals the identification of a novel subset of mDA neurons in the VTA that are marked by the expression of the bHLH transcription factor NEUROD6. *Neurod6*+ VTA neurons express a combination of molecular markers including OTX2, ALDH1A1, CALBINDIN1 and *Grp* and project specifically to the lateral septum region of the forebrain.

Despite increasing evidence of heterogeneity among mDA neurons, there is still a paucity of specific markers to identify distinct mDA neuron subsets. mDA neurons in the VTA have been subdivided into molecularly distinct subsets based on combinational expression of more broadly expressed genes (Di Salvio *et al*., 2010a). For example, OTX2, CALBINDIN1 and ALDH1A1 mark a ventral subset of VTA neurons, while OTX2+/CALBINDIN1+ and OTX+/GIRK2+ label central and dorsal VTA neurons, respectively. Our molecular characterisation shows that the ventral VTA subset can be further subdivided by the expression of *Neurod6*. Recent studies using single-cell transcriptome profiling have also identified a molecularly distinct subset of mDA neurons in the VTA that co-expresses *Otx2*, *Aldh1a1*, *Calb*, *Grp*, *Lpl* and *Adcyap1* (Poulin *et al*., 2014). Since *Neurod6*+ mDA neurons also express the first four of these markers, it is probable that *Neurod6* marks the same subset. *Neurod6* is however uniquely expressed in this neuronal subset, in contrast to OTX2, CALBINDIN1 and ALDH1A1, which all have a broader expression in mDA neurons (Di Salvio *et al*., 2010a; Poulin *et al*., 2014).

Molecular characterisation of *Neurod6*+ mDA neurons, including their expression of the neuropeptide *Grp* that is implicated in the regulation of memory associated with fear and emotional arousal, social interaction and food intake (reviewed in (Roesler and Schwartsmann, 2012), will aid in classifying these neurons and studying their potential functions in regulating memory and emotional behaviours.

Chapter 4. *Neurod6* is required for the survival of mDA neurons projecting to the LSi

4.1 Introduction

NEUROD6 also specifies the fate of a subtype of retinal amacrine cells (Cherry *et al*., 2011; Kay *et al*., 2011) and it has been implicated in the survival of cultured rat pheochromocytoma PC12 cells, where it enhances mitochondrial biogenesis and regulates cytoskeletal organization (Uittenbogaard and Chiaramello, 2005; Uittenbogaard *et al*., 2010; Baxter *et al*., 2012). Neurod6-mediated neuronal survival of these cells involves an increase in mitochondrial DNA copy number and content as seen by an increase in mitochondrial transcription factor A (TFAM) levels (Baxter *et al*., 2012). In addition, Neurod6 enhances the bioenergetics reserve of these neurons by increasing the levels of specific respiratory subunits (Baxter *et al*., 2012). Neurod6 is also able to increase mitochondrial membrane potential as visualized by increased fluorescence intensity of the TMRM dye (Baxter *et al*., 2012). Furthermore, work on PC12 cells demonstrate that Neurod6 is neuroprotective by making these neurons more resistant to oxidative stressors such as rotenone (Baxter *et al*., 2012). These important roles of NEUROD6 in protection and neuronal survival raised the possibility of similar roles for this factor in mDA neurons.

The A10-VTA mDA neurons project to several forebrain target regions. VTA mDA neurons projecting via the mesocortical pathway terminate their axons in the cortex; cingulate, prefrontal and perirhinal cortices. Other VTA mDA neurons project via the mesolimbic pathways to innervate various limbic structures including the amygdala, olfactory tubercle and the lateral septum.

A number of techniques have been used to identify axon target sites of mDA neurons in the VTA and SNc including both retrograde and anterograde tracing. Amongst the several conventional retrograde tracers that are available, fluorogold (FG; chemical name: hydroxystilbamidine) is particularly advantageous possessing several properties that make it suitable for retrograde tracing of mDA axons. FG is a fluorescent dye that emits different frequencies of light when bound to DNA and RNA. The beneficial properties of FG include that it has intense fluorescence and has high resistance to fading remaining in labelled neurons for long periods of time with a wide latitude of survival times ranging from 4 days to 2 months (Naumann *et al*., 2000). Additionally, it is detectable by conventional fluorescence microscopy using an ultraviolet excitation filter and can be photographed digitally. More importantly, FG can be injected into any PNS and CNS structure, it does not diffuse from labelled cells and is not taken up by intact undamaged fibers of passage although this was disputed in earlier studies it is now well established that only fibers terminating at the site of injection take up FG into their presynaptic terminals and retrogradely transport FG back to their cells of origin labelling their cell bodies with FG. Lastly, FG is compatible with all other histochemical techniques used in the study of the nervous system including immunohistochemistry making co-labelling experiments with other mDA molecular markers and retrograde-labelled mDA cells possible for the identification of targets sites of molecularly-defined subsets.

4.2 Results

4.2.1 *Neurod6* is required for the survival of a subset of VTA mDA neurons

To determine the role of *Neurod6* in mDA neurons, I analysed *Neurod6*$^{Cre/Cre}$; *R26R*$^{YFP/YFP}$ homozygous mutant mice. The total number and spatial distribution of *Neurod6*+ mDA neurons along the rostral-caudal axis of the VTA were evaluated by immunohistochemistry for TH and YFP at E18.5, postnatal day 3 (P3), P14 and 2 months (Figure 4-1A-H and data not shown). I focused the quantitative analysis on anatomically defined sections in the central mDA region (see Materials and Methods), which contain the highest numbers of Neurod6+ cells (approximately 50% of the total number of VTA Neurod6+ mDA neurons; Figure 4-1L-N) and displayed a loss of Neurod6+ mDA neurons. At E18.5, I did not observe a significant difference in the total numbers of YFP+/TH+ cell in the central mDA region between *Neurod6* mutant and control embryos (Table 4-1). At postnatal stages in contrast, there was a significant reduction in numbers of TH+/YFP+ mDA neurons in this region of *Neurod6* mutant compared to control mice (28% of control numbers at P3, 31% at P14 and 32% at 2 months; Figure 4-1 and Table 4-1). The VTA in adult mice can be further subdivided into several nuclei, including the IF, dorsal (dPN) and ventral paranigral nucleus (vPN), and PBP (Oades and Halliday, 1987) (Figure 4-1I). YFP+/TH+ cells were found in all these nuclei in *Neurod6* control mice (Figure 4-1J) and were missing specifically in the IF, dPN and PBP in *Neurod6* mutant mice (Figure 4-1K). Altogether, these results show that the deletion of *Neurod6* results in a loss of approximately 30% *Neurod6*+, TH+ mDA neurons in the central mDA region between

E18.5 and P3 that persists at adult stages. YFP+/TH+ mDA neurons that subsist in the absence of *Neurod6* are localised in the vPN and the PBP (Figure 4-1K).

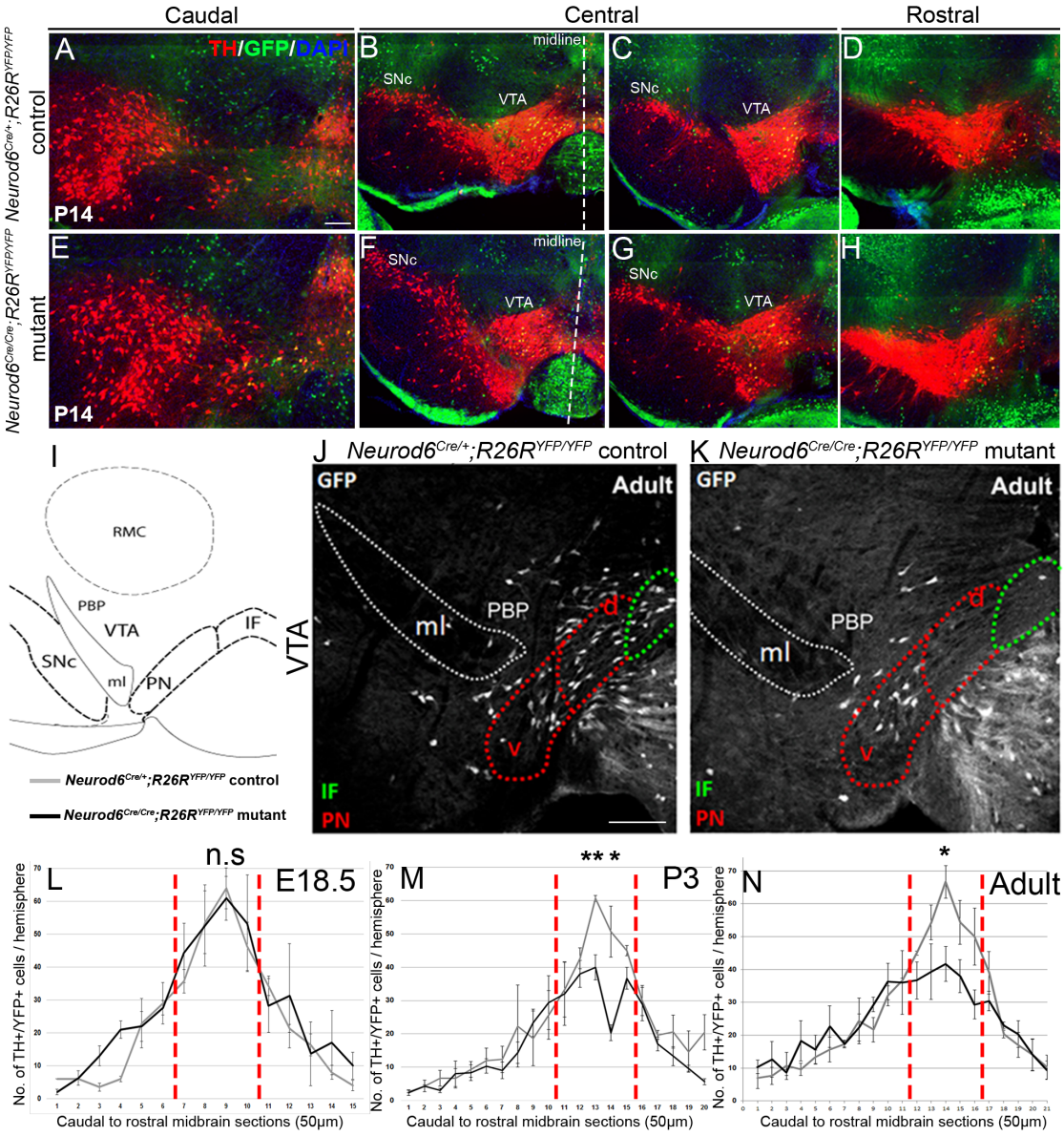

Figure 4-1 Partial reduction in the number of *Neurod6*+ mDA neurons in the absence of NEUROD6 function

A-H, Immunohistochemistry for both YFP and TH on coronal sections from the caudal to the rostral extent of the mDA region at P14. Reduced numbers of YFP+,TH+ neurons are observed in the central mDA region (B,C,F,G), while there is no apparent change in the numbers of YFP+TH+ mDA neurons in the rostral and caudal midbrain at P14. ***I***, Schematic diagram showing the positions of mDA nuclei in the VTA and anatomical landmarks. ***J,K***, Double antibody labelling of YFP and TH on a section through the central mDA region shows that YFP+TH+ neurons are lost mostly in the dorsal paranigral (PN), parabrachial (PBP) and interfascicular (IF)

nuclei (only the YFP channel is shown). ***L-N***, Graph showing the number of YFP+,TH+ mDA neurons analysed by immunohistochemistry on coronal midbrain sections from the caudal to the rostral extent of the mDA region at different stages. *Neurdo6*+ mDA neurons in the VTA are lost predominantly in the central mDA region corresponding to sections demarcated by red vertical lines. RMC; red nucles; ml, medial lemiscus; SNc, substantia nigra pars compacta; VTA, ventral tegmental area. Error bars denote SEM. * p<0.05; **p<0.01 (Student's t test). Scale bars: (A-H) 200μm, (J,K) 100μm.

	E18.5	P3	P14	Adult
n	3	3	3	3
Neurod6 Controls (*Mean* ± *S.E.M*)	200 ± 3	232 ± 5	207 ± 1	270 ± 16
Neurod6 Mutants (*Mean* ± *S.E.M*)	211 ± 27	167 ± 10	143 ± 14	185 ± 26
% of YFP+/TH+ mDA cells lost in mutants relative to controls	N/A	28%	31%	32%
Average number of cells lost in mutants (*Mean* ± *S.E.M*)	N/A	65 ± 14	65 ± 14	85 ± 15
significance	n.s	**	**	*
p-value	0.69	0.004	0.001	0.05

Table 4-1 Loss of YFP+/TH+ cells in the central mDA region of *Neurod6*$^{Cre/Cre}$;*R26R*$^{YFP/YFP}$ mutants per brain hemisphere

This table shows the raw data of the numbers of mDA neurons counted per brain hemisphere in the central mDA region and the results from statistical analysis, comparing cell counts between *Neurod6* mutant and control brains at each stage using the unpaired Student's t test. *n*, number of brain samples analysed; S.E.M, standard error of the mean; n.s, not significant.

Next, I asked whether the absence of *Neurod6*+ neurons in *Neurod6* mutants from P3 onwards was due to apoptosis, and I defined the precise timing of elimination of these cells, by conducting TUNEL analysis between E18.5 and P3. Many cells in the VTA of *Neurod6* mutant mice at P2 were TUNEL+ and expressed TH, while TUNEL+ cells were very rarely observed in control mice at this stage (Figure 4-2A), indicating that *Neurod6*+ VTA neurons die from apoptosis at early postnatal stages in the absence of *Neurod6*.

The mechanisms of *Neurod6*-mediated neuronal survival have been extensively studied in PC12 cells where *Neurod6* functions to protect against oxidative stress by sustaining mitochondrial mass (Uittenbogaard *et al*., 2010). To assess changes in mitochondrial mass prior to neuronal cell death in *Neurod6* mutant VTA neurons, I examined the expression of the mitochondrial import receptor subunit (TOM-20) at E18.5. TOM-20 expression level in YFP+/TH+ VTA neurons, measured by densitometric analysis, was significantly decreased in *Neurod6* mutant compared to control embryo (Figure 4-2B). A smaller but significant reduction in TOM-20 labelling was also observed in YFP- (*Neurod6*-)/TH+ mDA neurons (Figure 4-2B). These results indicate a robust reduction in mitochondria mass in both *Neurod6*+ and *Neurod6*- mDA neurons, and the difference between these two neuronal populations is also significant. Therefore, the sustenance of a subset of VTA neurons by *Neurod6* is likely to involve the maintenance of their mitochondrial mass.

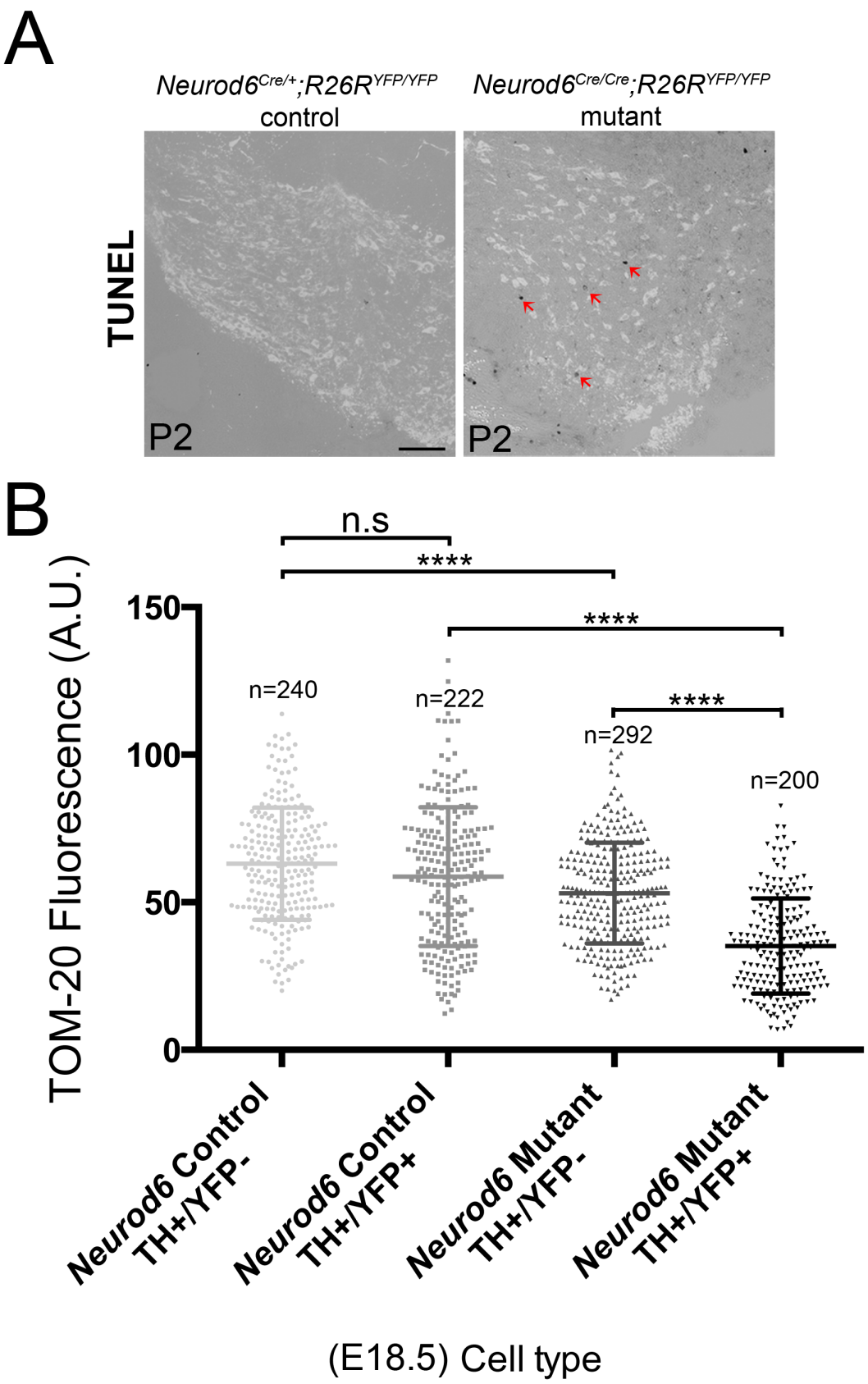
A
Neurod6Cre/+;R26RYFP/YFP
control
Neurod6Cre/Cre;R26RYFP/YFP
mutant
TUNEL
P2
P2
B
n.s
****
****
****
150
100
50
0
n=240
n=222
n=292
n=200
TOM-20 Fluorescence (A.U.)
Neurod6 Control TH+/YFP-
Neurod6 Control TH+/YFP+
Neurod6 Mutant TH+/YFP-
Neurod6 Mutant TH+/YFP+
(E18.5) Cell type

Figure 4-2 Loss of NEUROD6 function results in cell death and reduced mitochondrial mass in mDA neurons

A, Apoptotic cells (arrowheads) revealed by TUNEL analysis are observed in the TH+ mDA region of *Neurod6* mutant, but not in control pups at P2. ***B***, Reduction in mitochondria mass measured by the mean fluorescence (normalised to cell area) of neurons analysed by immunohistochemistry with Tom20, TH and YFP (AU, arbitrary units) in *Neurod6* control and mutant embryos at E18.5. n, number of neurons analysed. ** p<0.01; ****p<0.0001 (one-way ANOVA, with Tukey's post-test). Scale bar 200μm.

4.2.2 Specific changes in axon projections of mDA neurons to the lateral septum in adult Neurod6 mutant mice

The loss of a subset of VTA neurons in *Neurod6* mutant mice is likely to affect the connectivity between the VTA and other brain regions. To address this possibility, I first compared the axon projections of mDA neurons in adult *Neurod6* mutant and control mice by immunohistochemistry for TH. I analysed the neuronal projection targets of VTA neurons (A10) in the septum, prefrontal cortex, nucleus accumbens, amygdala and olfactory tubercle. In control mice, a dense arborisation of TH+ axons was observed in the intermediate lateral septum (LSi) and fine varicosities of TH+ mDA axons were found in the dorsal lateral septum (LSd) (Figure 4-3A,B). The dense arbor in the LSi was completely absent in *Neurod6* mutants, while the axons of the LSd were unaffected (Figure 4-3A,B). TH+ mDA axons in all other A10 neuronal target sites also appeared normal in *Neurod6* mutant mice (Figure 4-3E-J). The loss of both *Neurod6*+ neurons and TH+ axonal projections in *Neurod6* mutant mice suggests that *Neurod6*+ VTA neurons project to the lateral septum.

The specific loss of TH+ mDA axons in the LSi was also observed at P3, P7 and P14 (data not shown). I examined the septal target site at E18.5 before the loss of YFP+ mDA neurons in mutant embryos, however TH+ fibres were not detected in

the dorsal lateral septal areas of control embryos, suggesting that at this stage TH+ mDA axons have not yet reached this target site (Figure 4-3C,D).

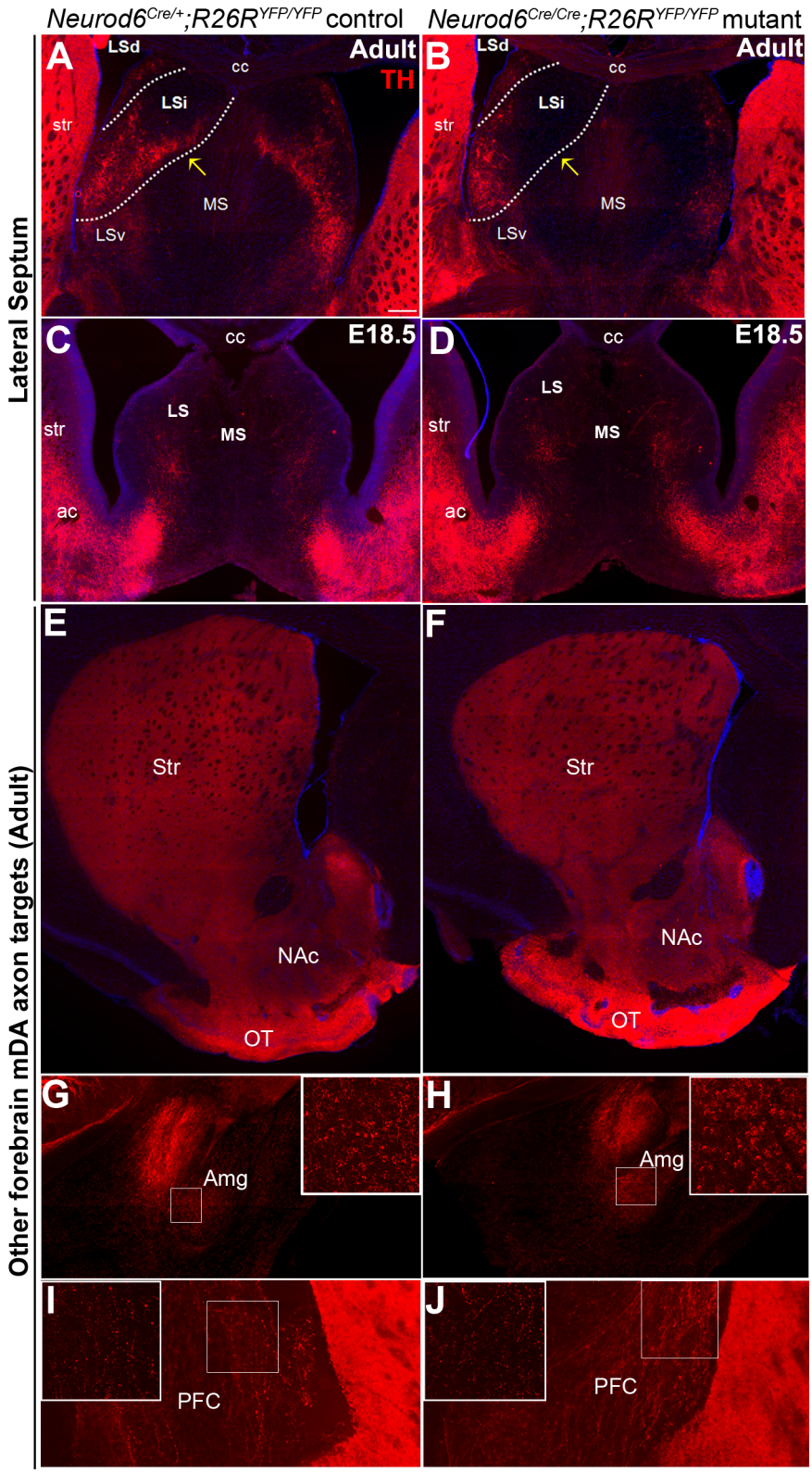

Figure 4-3 TH+ mDA axon projections to the intermediate region of the lateral septum are specifically missing in adult *Neurod6* mutant mice

A,B, TH immunohistochemistry show specific loss of axon projections to the intermediate region (Lsi) but not to the dorsal region (LSd) of the lateral septum in *Neurod6* mutant adult mice. ***C,D,*** TH+ axon projections from mDA neurons have reached the ventral region of the lateral septum (LS), but no TH+ fibres were found dorsally in the lateral septum of *Neurod6* mutant embryos at E18.5. ***E-J***, TH+ mDA neuronal projections to other target sites of the VTA including nucleus accumbens (NAc), olfactory tubercle (OT), amygdala (Amg) and prefrontal cortex (PFC) appear normal in *Neurod6* mutant compared to control adult mice. TH+ axons of SNc mDA neuronal projections to the striatum (Str) were also unaffected. MS, medial septum; LSv, lateral septum, ventral; MS, medial septum; cc, corpus callosum; ac, anterior commissure. Insets in ***G-J*** show higher magnification of boxed region. Scale bar 200μm.

4.2.3 Fluorogold retrograde labelling demonstrates that Neurod6+ mDA neurons project to the lateral septum

Next, to determine whether the lateral septum is a specific target site for *Neurod6*+ mDA neurons, collaborators performed fluorogold (FG) retrograde labelling experiments. FG was injected into the lateral septum of *Neurod6* control and mutant pups at P10 and retrograde transport of FG into cell bodies of mDA neurons was analysed at P13 (Figure 4-4A). I first confirmed that FG had been specifically injected into the LSd and LSi regions of the septum by staining for BDA, which was co-injected with FG. Coronal sections of the forebrain at the level of the septum showed BDA+ fibres in the lateral septal region, but not in the adjacent striatal region (Figure 4-4B). In the midbrain of *Neurod6*+ control mice at P13, FG labelled most YFP+/TH+ mDA neurons in the IF, dPN, vPN and PBP of the VTA, demonstrating that *Neurod6*+ mDA neurons are lateral septal-projecting neurons (Figure 4-4C). In *Neurod6* mutant mice, intra-septal FG injections retrogradely labelled many of the YFP+/TH+ mDA neurons that remain in the vPN and PBP of the VTA, indicating that these neurons project to the LSd and their axons likely correspond to the remaining TH+ fibres in

the LSd of *Neurod6* mutants (Figure 4-4D). Altogether, these results suggest that the YFP+/TH+ mDA neurons missing in *Neurod6* mutant animals project to the LSi, while the remaining YFP+/TH+ cells in these mutants project to the LSd.

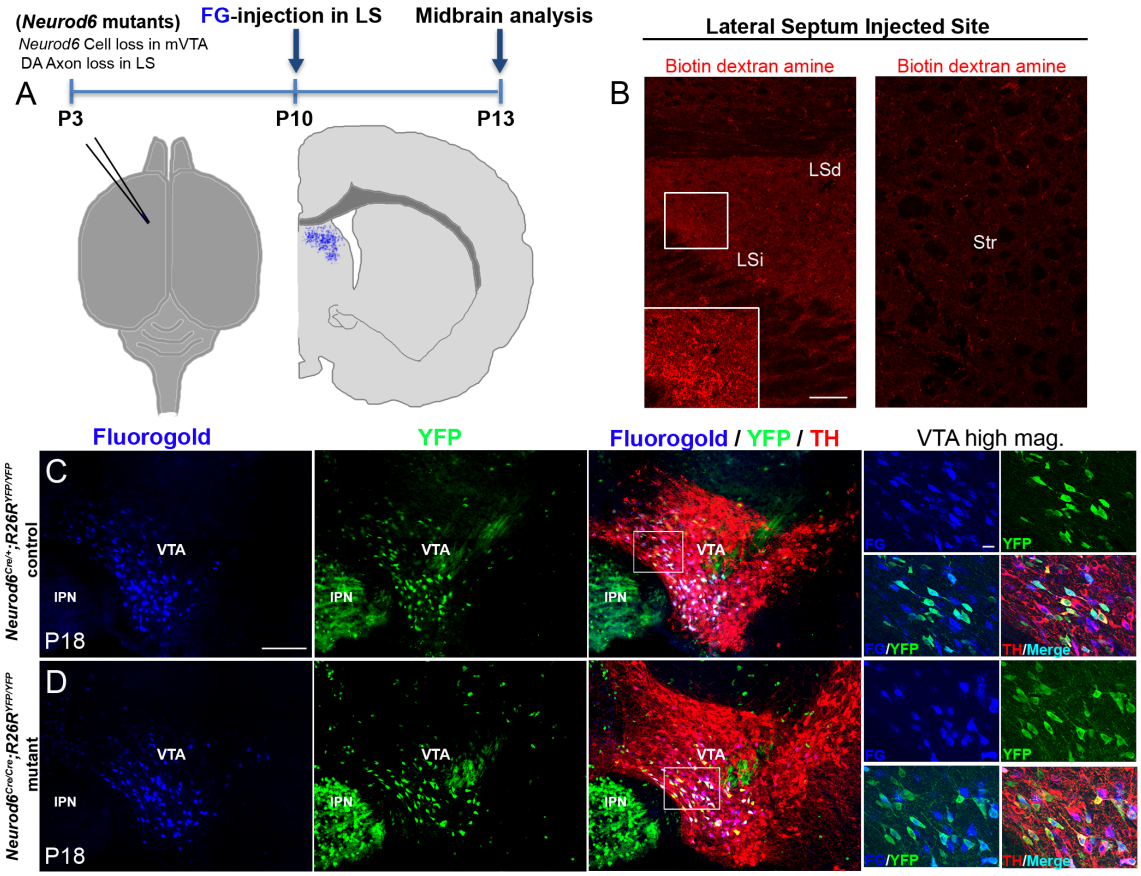

Figure 4-4 Fluorogold retrograde labelling experiments show that Neurod6+ mDA neurons project to the dorsolateral and intermediate region of the lateral septum

A*,** Schematic diagram indicating the position of fluorogold injection in the injected brain and the schedule of the experiment. ***B*,** Biotin-dextran is detected specifically in the intermediate region (LSi) as well as the dorsal region (LSd) of the lateral septum but not in the adjacent striatal regions. ***C, D. Injection of fluorogold into the septal region results in its retrograde transport of fluorogold into YFP+ TH+ mDA cell bodies in *Neurod6* control (C) and mutant (D) mice at P18. Scale bars: (B) 100μm, (C,D) 200μm, high magnification panels 20μm.

4.3 Discussion

In the previous chapter, I reported the identification of a novel subset of VTA neurons marked by the expression of *Neurod6* and the molecular characterisation of this subset. In this chapter, I have examined the role of Neurod6 in the survival of mDA neurons by analysing Neurod6 mutants as well as controls. My analysis revealed a 30% reduction in the number of *Neurod6*+ mDA neurons in the VTA of *Neurod6* single mutant mice at P3, and this phenotype was maintained in adult mutant mice. Loss of these neurons was accompanied by loss of TH+ fibres in the LSi of *Neurod6* single mutants, indicating that *Neurod6* alone is required for the survival of LSi-projecting mDA neurons.

My anatomical studies have demonstrated that *Neurod6*+ mDA neurons are distributed in the PBP, PN and IF nuclei of the adult VTA. These neurons are found in similar numbers and with comparable spatial distributions along the rostral-caudal and medial-lateral axis of the midbrain from E18.5 to adult stage, suggesting that *Neurod6* expression is established in mDA neurons during embryogenesis and persists through adulthood (Table 4-1). My findings therefore provide evidence for a developmental heterogeneity of mDA neurons that likely has implications for their adult function and for homeostasis.

NEUROD6 has been shown to have a neuroprotective role in PC12 cells serum deprived or treated with the mitochondrial stressor rotenone, and to act by enhancing mitochondrial biogenesis (Uittenbogaard *et al*., 2010; Baxter *et al*., 2012). Consistent with these findings, I observed a decrease in mitochondria mass before the death of

Neurod6 mutant mDA neurons, suggesting that defects in energy metabolism contribute to the apoptosis of these neurons. Alternatively, changes in mitochondria mass might be an indirect consequence of neurons undergoing apoptosis because of the absence of anti-apoptotic factor regulated by NEUROD6. Support for the latter mechanism comes from the finding that mitochondria mass was decreased in both YFP-negative (*Neurod6*-negative) as well as YFP+ mDA neurons (Figure 4-2B) and from the report that Neurod6 regulates the expression of anti-apoptotic factors in PC12 cells (Uittenbogaard and Chiaramello, 2005).

Fluorogold retrograde tracing experiments established that *Neurod6*+ neurons project to the lateral septum. In this region, *Neurod6*+ mDA neurons project to both the LSi and LSd. Other VTA neurons also project to the lateral septum, since some of the mDA neurons labelled by fluorogold did not express *Neurod6*. Consistent with earlier studies in the rat, TH+ axon projections to the septum become established at early postnatal stages since the TH+ fibres were observed in P3 pups and not in E18.5 embryos (Antonopoulos *et al*., 1997). The role of dopamine in the lateral septum is poorly studied. Lesions of septal dopaminergic terminals by injection 6-hydroxydopamine into the lateral septum of rats result in deficits in spatial memory tasks (Simon *et al*., 1986).

Chapter 5. *Neurod6* and *Neurod1* regulate the survival of LS-projecting mDA neurons

5.1 Introduction

NEUROD6 belongs to the NEUROD subfamily of bHLH transcription factors, which consists of 4 members including NEUROD1, NEUROD2, NEUROD4 and NEUROD6 (Bertrand *et al*., 2002). Functionally redundant and compensatory roles by members of the NeuroD family of proteins is possible due to firstly, the high sequence similarity within their bHLH domains that are required for DNA binding and dimerisation and secondly, the highly overlapping expression profiles of NeuroD family members across several brain regions. To study functional redundancy and compensation between more than one NeuroD factor, simultaneous inactivation of more than one member has been conducted. The phenotypes of double mutants have been compared to those of individual single gene inactivation.

Since Neurod6 single mutants generated by Schwab *et al*., (1998) did not exhibit any obvious abnormalities (Schwab *et al*., 1998), they generated Neurod1/6 double mutants (Schwab *et al*., 2000) since these two factors exhibited overlapping expression patterns within the neocortex, hippocampus and cerebellum (Schwab *et al*., 1998; Schwab *et al*., 2000). Although Neurod1/6 double mutant mice died shortly after birth due to Neurod1-deficiency causing pancreatic failure, Neurod1/6 double mutants revealed redundant functions of these two factors specifically in the terminal neuronal differentiation of hippocampal granule cells (Schwab *et al*., 2000).

Neurod2/6 double-deficient mice were generated by Bormuth *et al*., (2013) to study

their redundant roles in cortical commissure formation. Neurod2/6 double mutant mice exhibited several defects in late neuronal functions including disorganization of dendritic morphology, reduction of glutamatergic synapses and impairment of commissural tract formation (Bormuth *et al*., 2013). Together, these findings demonstrated that NEUROD2 and NEUROD6 are both required for the fasciculation and directional growth of callosal axons in the mouse neocortex (Bormuth *et al*., 2013).

This far, I have reported the expression and role of Neurod6 in the survival of a subset of mDA neurons. However, many brain regions usually express more than one bHLH member of the NeuroD family of transcription factors. Furthermore in the absence of Neurod6, only ~30% of the Neurod6-subset of mDA neurons as seen by YFP expression are lost in *Neurod6*$^{Cre/Cre}$*;R26R*$^{YFP/YFP}$ mutants. Together this could indicate towards the possibility of another bHLH member of the NeuroD family being expressed within this mDA subset with potential compensatory roles for the survival functions of Neurod6 in the remaining surviving YFP neurons of Neurod6 mutants.

5.2 Results

5.2.1 *Neurod1* is broadly expressed by mDA neurons

Since *Neurod* family members share redundant roles in other parts of the nervous system, we next addressed the possibility that another *Neurod* gene promotes the survival of a subset of *Neurod6*+ neurons in *Neurod6* mutants. We first examined the expression profiles of NEUROD1 and NEUROD2 in mDA neurons. *Neurod1* transcripts were detected by *in situ* hybridisation strongly in immature and weakly in mature mDA neurons at E13.5 and E14.5 (Figure 5-1A,B). NEUROD1 expression analysed by immunohistochemistry of $Neurod6^{Cre/+;}Rosa26R^{YFP/YFP}$ embryos was maintained in all mature TH+ mDA neurons including YFP+ (*Neurod6+)* mDA neurons at E18.5 (Figure 5-1C). In addition, in situ hybridization of *Neurod1* followed by immunohistochemistry of TH showed that this expression was maintained into the adult stage (Figure 5-1D). In contrast, NEUROD2 analysed by immunohistochemistry, was not expressed in mDA neurons at E18.5 (Figure 5-2A,B).

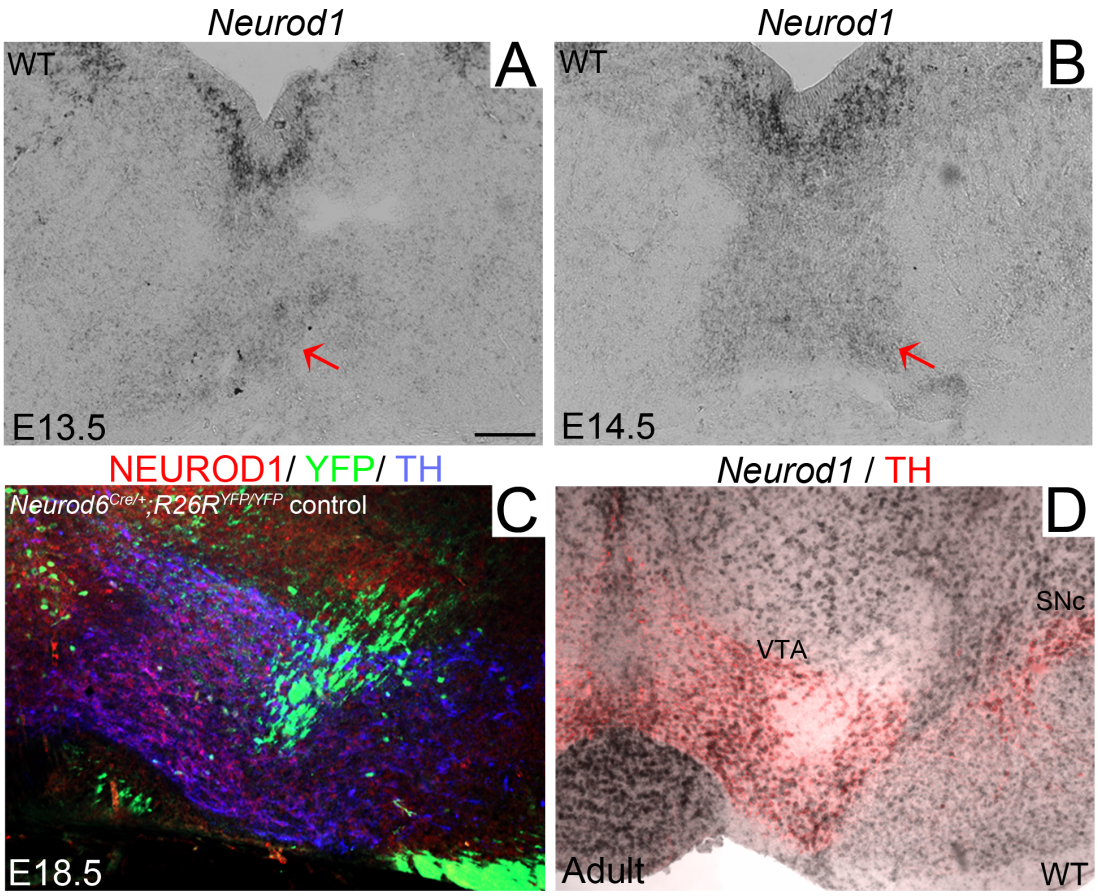

Figure 5-1 Neurod1 is expressed in mDA neurons

A,B, In situ hybridization show that *Neurod1* transcripts are expressed strongly and weakly respectively in immature and mature (arrows) in mDA neurons located ventral to the floor plate of the midbrain at E13.5 (**A**) and E14.5 (**B**). ***C,D,*** This expression, detected by triple immunolabeling with NEUROD1, TH and YFP antibodies, is maintained in mature YFP+ and TH+ mDA neurons at E18.5 and in adult *Neurod6* control mice by in situ hybridization of *Neurod1* followed by immunohistochemistry of TH. Scale bar 100µm.

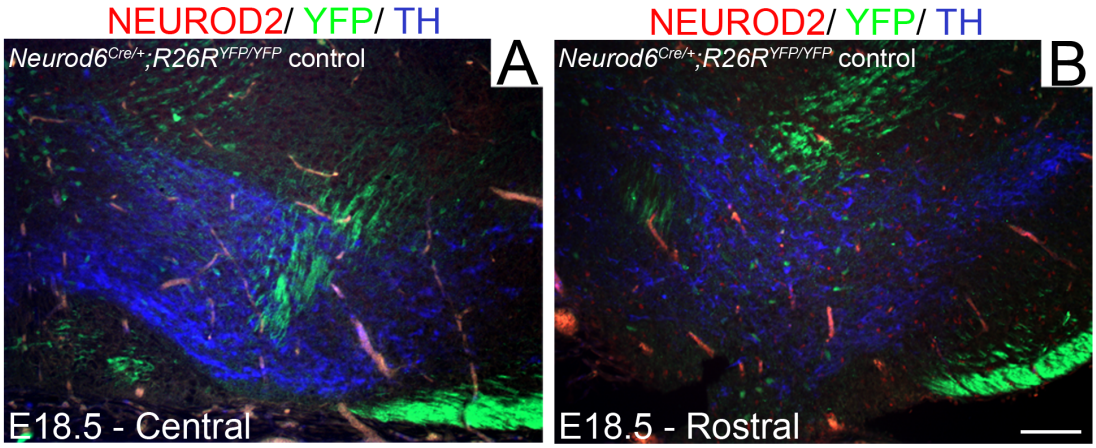

Figure 5-2 Neurod2 is not expressed in mDA neurons

A, NEUROD2 was not detected in mDA neurons and, ***B,*** in only sporadic TH- cells at E18.5 by immunohistochemistry in sections of central (**A**) and rostral (**B**) mDA regions. Scale bar 100μm.

5.2.2 Severe loss of Neurod6+ VTA neurons in Neurod1 and Neurod6 double mutants

To determine whether *Neurod1* is required for the survival of *Neurod6*+ mDA neurons, we used mice carrying a null allele of *Neurod1* whereby *LacZ* replaces the *Neurod1* coding sequence (Naya *et al*., 1997; Miyata *et al*., 1999) to generate *Neurod1* and *Neurod6* (*Neurod1;Neurod6*) double mutant mice (*Neurod1*$^{LacZ/LacZ}$;*Neurod6*$^{Cre/Cre}$;*R26R*). The double homozygous mutants died shortly after birth at P0, as the *Neurod1*$^{LacZ/LacZ}$ single mutants that die from neonatal diabetes (Miyata *et al*., 1999). We therefore examined *Neurod6*+ VTA neurons at E18.5 and observed a severe loss of YFP+/TH+ mDA neurons in the VTA of *Neurod1;Neurod6* double homozygous mutants (Figure 5-3F,G) compared to both control (Figure 5-3A,G) and *Neurod6*$^{Cre/Cre}$ single mutant embryos (Figure 5-3B,G), which do not show a cell loss at this stage. Moreover, mice homozygous mutant for *Neurod1* and heterozygous for *Neurod6* (*Neurod1*$^{LacZ/LacZ}$;*Neurod6*$^{Cre/+)}$ as well as

mice homozygous mutant for *Neurod6* and heterozygous for *Neurod1* (*Neurod1*$^{LacZ/}$*+;Neurod6*$^{Cre/Cre}$*)* and double heterozygous (*Neurod1*$^{LacZ/+}$*;Neurod6*$^{Cre/+}$*)* mice also present significant losses of YFP+/TH+ mDA neurons compared to *Neurod6*$^{Cre/Cre}$ single mutants at E18.5 (Figure 5-3D-F respectively and Figure 5-3G). These results suggest that both *Neurod1* and *Neurod6* contribute to the survival of VTA *Neurod6*+ neurons before birth, since loss of one copy of each gene reduces neuronal viability. They also suggest that *Neurod1* has a more important role than *Neurod6* in *Neurod6*+ neuron survival, since loss of one copy of *Neurod1* and one copy of *Neurod6* results in the loss of some neurons while loss of two copies of *Neurod6* has no effect at E18.5. However, the early postnatal death of double mutant mice precluded the analysis of mDA neurons and axonal projections at later stages. Earlier analysis of double mutant embryos was also not feasible since YFP, which serves to mark *Neurod6*+ mDA neurons, only becomes detectable at E18.5 (data not shown).

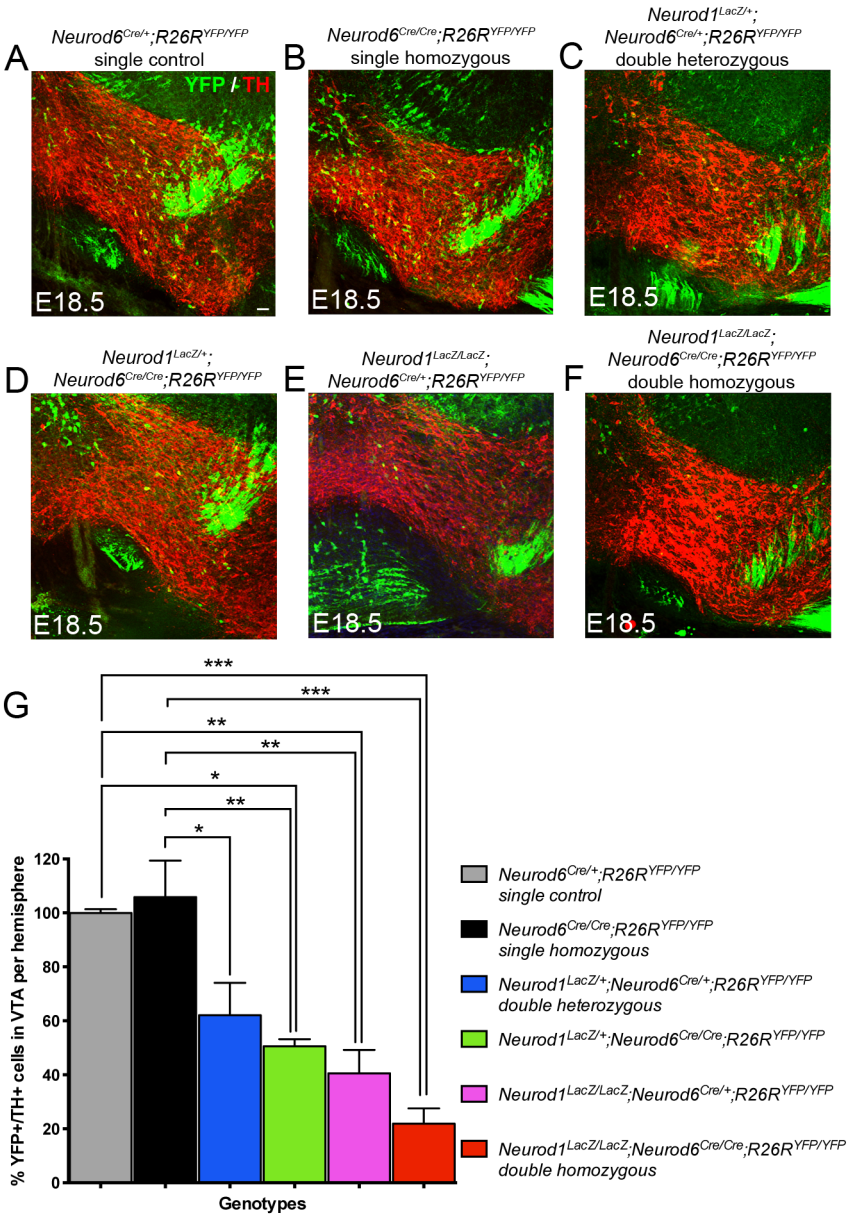

Figure 5-3 Neurod1 is also required for the survival of Neurod6+ mDA neurons.

A-F, YFP+,TH+ mDA neurons are lost at E18.5 in *Neurod1;Neurod6* double mutants carrying different copy numbers of *Neurod1* and *Neurod6* mutant alleles. Immunohistochemistry of YFP and TH shows that reduced numbers of YFP+/TH+ mDA neurons are observed in coronal sections of the central midbrain in double mutant embryos carrying 2 (***C***), 3 (***D,E***) or 4 *Neurod1;Neurod6* mutant alleles (***F***). In contrast, *Neurod6* single homozygous mutants present no change in YFP+/TH+ expression in mDA neurons at E18.5 (***B***). ***G***, Bar graph showing percentage changes of the number of YFP+/TH+ mDA neurons in the central mDA region of *Neurod1;Neurod6* double and *Neurod6* single mutant embryos normalised to *Neurod6*$^{Cre/+}$ control embryos. Error bars denote SEM. N=3 per genotype.

*p<0.05; **p<0.01; ***p<0.0001 (one-way ANOVA, with Tukey's post-test). Scale bar 100μm.

5.3 Discussion

Genetic studies have revealed that *Neurod6* alone is required for the survival of LSi-projecting *Neurod6*+ mDA neurons postnatally (see previous chapter), while the survival of *Neurod6*+ mDA neurons are dependent on both *Neurod1* and *Neurod6* embryonically as demonstrated in this chapter. Together, these results have revealed essential roles for NEUROD-family proteins in regulating the survival of this mDA neuronal subset.

Since NEUROD1 is also expressed in both immature and mature mDA neurons during development and since *Neurod6*+ mDA neurons are partially lost in *Neurod6* single mutants, we also analysed the phenotypes of *Neurod1;Neurod6* double mutant mice. Severe loss of *Neurod6*+ mDA neurons occurred in double homozygous mice at E18.5, i.e. before the cells are lost in *Neurod6* single mutants. *Neurod6*+ mDA neurons were also lost in double heterozygous mice and in mice homozygous for the *Neurod1* mutation and heterozygous for the *Neurod6* mutation. Because of the unavailability of NEUROD6-specific antibodies, we cannot determine the status of *Neurod6*+ mDA neurons in *Neurod1* single mutants. Together, our genetic analysis demonstrates that both *Neurod6* and *Neurod1* contribute to the survival of *Neurod6*+ mDA neurons. It also suggests that *Neurod1* has a more important role than *Neurod6* for *Neurod6*+ mDA neuronal survival at E18.5 since loss of the two copies of *Neurod6* has no effect while loss of one copy of *Neurod1* and one copy of *Neurod6* reduces survival of these neurons.

Further studies will be required to identify the mechanisms through which NEUROD family proteins regulate the survival of embryonic mDA neurons during development and to determine whether these proteins continue to have a survival role in these neurons during adult life.

Chapter 6. Discussion

DA neuron diversity is a challenging field of immense importance for understanding DA related neuropsychiatric disorders. The work presented in this thesis describes a subset of DA neurons, defined by a new marker Neurod6. I have characterised the expression of this marker, correlated it with other markers, and demonstrated that these neurons project to the septum. I then determined that in mice lacking NeuroD6, this subset is partially lost. This mild phenotype is exacerbated in *Neurod1;Neurod6* double mutants.

This study investigates the role of bHLH protein NEUROD6 in the development of a subset of DA neurons in the VTA of the ventral midbrain. Using i*n situ* hybridisation, RNAscope-TH double labelling and *Neurod6*$^{Cre/+}$*;R26R*$^{YFP/YFP}$ mice, I have shown that NEUROD6 is indeed co-expressed in a subset of DA neurons in the VTA, which co-express OTX2, CALBINDIN and ALDH1A1. I have then used *Neurod6*$^{Cre/Cre}$*;R26R*$^{YFP/YFP}$ mutant mice to show that loss of NEUROD6 reduces 30% of Neurod6+ mDA neurons in VTA, due to an increase in cell death. Using TH staining and FG retrograde labelling, I have postulated that the Neurod6+ mDA neurons projected their axons to specific regions in the LS. Finally, I have demonstrated that removing NeuroD1, further eliminated all the NeuroD6+ neurons in the VTA, suggesting functional redundancy of NeuroD1 in promoting the survival of NeuroD6+ mDA neurons.

6.1 Neurod6 defines a new VTA-specific mDA subset

In the analysis of YFP-labelled Neurod6+ mDA neurons in *Neurod6* controls, I have defined the localisation of these neurons to the ventral midbrain in specific VTA nuclei mostly in the dorsal and ventral components of the PN and IF as well as some in the lateral PBP (Chapter 3). Furthermore, I have molecularly identified these Neurod6+ mDA neurons with other known molecular markers of the VTA and have demonstrated that majority of Neurod6+ neurons define a new subset of Otx2+, Calbindin1+, Aldh1a1+, Nolz1+ and *Grp*+ VTA mDA neurons.

Single-cell gene profiling of global mDA neurons using microfluidic dynamic array technology followed by RT-qPCR of 96 genes revealed 6 unique mDA subtypes (Poulin *et al*., 2014). Principle component analysis grouped these 6 mDA subtype expressing combination of key genes into DA^{1A}, DA^{1B}, DA^{2A}, DA^{2B}, DA^{2C}, and DA^{2D} subtypes (Poulin *et al*., 2014). According to these gene expression profiles, Neurod6 likely belongs to the DA^{2B} subtype of mDA cells based on their expression of Otx2 and Aldh1a1. In addition to these markers, I have shown that Neurod6+ mDA neurons also co-express Grp and Nolz1 suggesting that the DA^{2B} subtype is a VTA-specific mDA subtype with a more complete molecular signature: Otx2+, Aldh1a1+, Nolz1+, Grp+ and Neurod6+ VTA neurons. Other molecular markers detected in this DA^{2B} subtype also includes Lpl (Poulin *et al*., 2014), supporting the possibility that Lpl is also expressed in Neurod6+ mDA neurons. Co-localisation of YFP+ cells in Neurod6 controls with Adcyap1 would further support this molecular identity of the DA2B subtype and support Neurod6 as a new molecular marker for this mDA subtype.

Aldh1a1+ VTA mDA neurons synthesise GABA, independent of GAD, for co-release with DA (Kim *et al*., 2015). Since Neurod6+ VTA neurons are part of the Aldh1a1+ mDA subset and co-localise with ALDH1A1, it remains to be determined whether Neurod6+ VTA neurons are able to synthesise and co-release both DA and GABA in the LS.

More importantly, the DA^{2B} mDA subtype described by Poulin *et al*., (2014), which likely includes Neurod6, is shown to be resistant to MPTP-treatment further supporting neuroprotective functions for VTA DA^{2B} molecular signature markers (Poulin *et al*., 2014).

Given that the Neurod6 VTA subset can fundamentally be divided into two subsets; one requiring Neurod6, the other requiring both Neurod6 and Neurod1 for survival, it is likely that this Neurod6-marked mDA subset represents two unique subsets with differential expression of molecular proteins in addition to distinct axon target sites (LSi and LSd) and their differential requirements for Neurod6 and Neurod1 bHLH proteins. Single-cell analysis of this Neurod6 subset may elucidate differentially expressed genes between LSi- and LSd- projecting Neurod6+ VTA neurons.

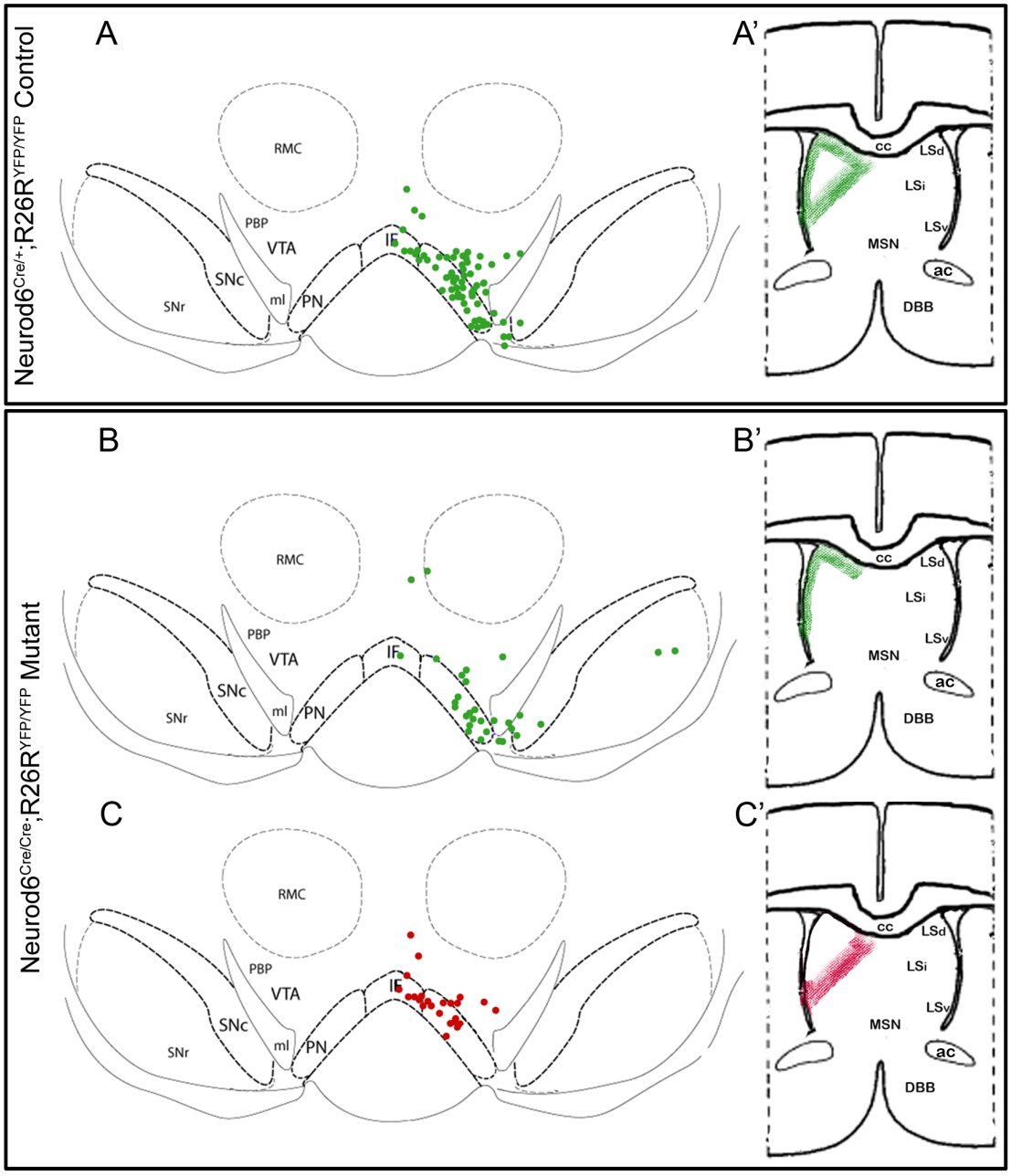

Figure 6-1 Summary of *Neurod6*+ mDA neurons projecting to LS and differential requirements for their survival.

Neurod6+ mDA neurons of the VTA project to the dorsal and intermediate components of the LS (A). Neurod6 is not required for the survival of LSd-projecting mDA neurons which survive in *Neurod6* mutants probably due to compensation by Neurod1 (B). Neurod6 is only required for the survival of LSi-projecting mDA neurons which are lost in *Neurod6* mutants (C). In the absence of both Neurod6 and Neurod1, both subsets are lost (not shown).

6.2 The LS in the forebrain is a projection target of Neurod6+ VTA mDA neurons

Several studies have described DA innervations in the LS (Lindvall, 1974; Onteniente *et al*., 1984; Lindvall and Stenevi, 1978) originate from the VTA (Assaf and Miller, 1977; Lindvall *et al*., 1977; Carter and Fibiger, 1977; Fallon and Moore, 1978). However, the work reported in this thesis is the first to molecularly identify this VTA subset(s) projecting to discrete regions within the LS – both the LSi and LSd – with their specific expression of the Neurod6 bHLH transcription factor protein. Furthermore, the requirements for NeuroD proteins is different between LSi and LSd-projecting VTA neurons. In summary, my results show that NeuroD6-expressing mDA neurons are localised within the medial VTA in specific nuclei and project to both the LSd and LSi. In the absence of NeuroD6, ~30% cells die within the dorso-medial PN component of the VTA, which correlates with mDA axon loss within the LSi. These neurons require NeuroD6 for their survival. Furthermore, in the absence of Neurod6, YFP cells in ventro-lateral PN component of the VTA survive & these project to the LSd. These neurons do not require Neurod6 for survival probably due to compensation by Neurod1. In the absence of both Neurod1 and Neurod6 in double mutants, most of this Neurod6-marked subset is lost. Over several years of DA research, it seems the LS is an mDA projection target has not been extensively studied at the functional level. Here is the first report of a target-specific (LS), molecular marker- defined (Neurod6) functional mDA subset. The next implication of this subset is elucidating its behavioural function and relevance to neurodevelopmental and neuropsychiatric disorders of the limbic system, which is beyond the scope of this thesis.

It remains to be determined whether Neurod6+ mDA neurons projecting to the LS, also send axon collaterals to other regions within the forebrain. VTA neurons of the PN can project their axons exclusively to the LS (Aransay *et al*., 2015) or send axonal branching to NAc as detected by single-cell axon tracing methods (Aransay *et al*., 2015). There were no observed changes in TH+ antibody labelling of mDA axons in any other forebrain targets other than the LS in Neurod6 mutants. TH+ immuno-labelling of axons did not show any obvious change in projections to the NAc in Neurod6 mutants compared to controls suggesting Neurod6+ VTA neurons from the PN project exclusively to the LS. However, if these axons do send collaterals to NAc, these may not be obvious. To address this, *Cre*- dependent anterograde tracing of Neurod6+ mDA axons could identify if there is also axonal branching to NAc regions. The findings that Otx2 is required for the upregulation of axon guidance molecules in VTA mDA neurons as well as loss of mDA axons in several VTA-specific forebrain target areas including both the LSd and LSi of Otx2 cKO mice (Chung *et al*., 2010), suggests the requirement of Otx2 function in LS-projecting Neurod6+ Otx2+ VTA neurons. The precise mechanisms by which Otx2 establishes correct projection targeting patterns of VTA neurons and whether this is independent of other transcription factors in VTA subsets is not understood. In addition to Neurod6's role in mDA neuronal survival, whether it has any additional roles in regulation of axon guidance of VTA neurons to the LS is unknown. Furthermore, Neurod2/6 regulate commissural formation in the cortex by regulating axon guidance molecules (Bormuth *et al*., 2013). Future experiments could investigate additional roles for Neurod1/6 in mDA axon guidance to the LS aside from their role in their survival.

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6.3 Candidate mechanisms for Neurod6-mediated neuronal survival of VTA neurons

In the absence of Neurod6, approximately 30% of VTA neurons are lost in *Neurod6* mutants shortly after birth at P2 due to cell death as seen by TUNEL analysis (Chapter 4). Determining the mechanism of Neurod6-mediated neuronal survival of mDA neurons of the VTA will facilitate our understanding of multiple pro- neuronal survival/ anti-apoptotic pathways. This could shed light on how the presence of these factors may make VTA mDA neurons more resilient to degeneration, in contrast to the absence of these factors, in SNc neurons, which are more susceptible to cell death.

6.3.1 PI3K/Akt signalling

Calbindin-D28K is a neuroprotective factor for CB-positive VTA neurons (Sun *et al*., 2011) compared to CB-negative SNc neurons that are more susceptible to degeneration in PD (German *et al*., 1992). The neuroprotective effects of CB on VTA mDA neurons is mediated by the activation of PI3-Kinase-AKT signalling pathway (Sun *et al*., 2011). PI3K/Akt signalling promotes survival by generation of phosphatidylinositol 3,4,5-triphosphate by PI3K, which activates Akt by phosphorylation of Ser-473 (Alessi *et al*., 1996). Activated Akt is able to influence the activity of downstream factors including caspase 9 and CREB thereby promoting neuronal survival (Datta *et al*., 1999). It is possible that Calbindin-D28K and Neurod6 act on the same pathway to inhibit apoptosis in VTA neurons and provide neuroprotective roles.

The Aldh1a1 enzyme synthesises RA in neurons. ALDH1A1 expression in mDA progenitor cells and mature VTA neurons in the adult begs the question of the role of RA in early and late mDA development and in Aldh1a1+/Neurod6+ VTA neurons. In a human neuroblastoma cell differentiation model, RA induces neural differentiation by modulation of bHLH factors (Lopez-Carballo *et al*., 2002). Specifically, RA down-regulates ID1, ID2 and ID3 genes that usually inhibit neural differentiation and down-regulates the neuroblastoma marker Mash1 (Lopez-Carballo *et al*., 2002). In contrast, RA induces an increase in mRNA levels of neural differentiation-promoting bHLH factors Neurod6 and Neurod1 (Lopez-Carballo *et al*., 2002) by rapid activation of the PI3K/Akt signalling pathway (Lopez-Carballo *et al*., 2002). Firstly, this may implicate early roles for Aldh1a1 in mDA progenitors for RA-activation of neural differentiation by induction of pro-differentiation factors such as Neurod1 (onset of expression strongly in immature and weakly in mature TH+ neurons at E13.5) and Neurod6 (onset of expression in already mature TH+ neurons at E15.5). Secondly, a late role of Aldh1a1 may be in promoting neuronal survival of Neurod6+/Aldh1a1+ VTA neurons via RA-induced activation of the PI3K/Akt signalling pathway. Neurod6 may be acting on this same pathway to mediate neuronal survival or independently of Akt via regulation of mitochondrial proteins.

6.3.2 Regulation of mitochondrial pathways

The mechanisms of Neurod6-mediated neuronal survival have been extensively studied in an established Neurod6-overexpressing rat PC12 pheochromocytoma cell line model system (Uittenbogaard and Chiaramello, 2005). Neurod6-mediated survival of PC12 cells is independent of PI3K/Akt signalling since Neurod6-PC12

cells fail to express phosphorylated forms of Akt in a serum deprivation experimental cell death paradigm (Uittenbogaard and Chiaramello, 2005). Instead, Neurod6 activates an anti-apoptotic program at two distinct regulatory levels of the mitochondrial pathway to promote neuronal survival by inducing the expression of the Bcl-w and XIAP anti-apoptotic pro-survival proteins (Uittenbogaard and Chiaramello, 2005). Neurod6 induces mitochondrial biogenesis and the expression of bioenergetics-related genes upon Neurod6 onset of expression during early stages of neuronal differentiation (Baxter *et al*., 2012). Furthermore, Neurod6 increases the mitochondrial membrane potential and ATP levels and confers tolerance to oxidative stress (Baxter *et al*., 2012). A working model of Neurod6-mediated neuronal survival via regulation of mitochondrial functions was published by Uittenbogaard *et al*., (2010) and is referenced here (Figure 6-2; Uittenbogaard *et al*., 2010).

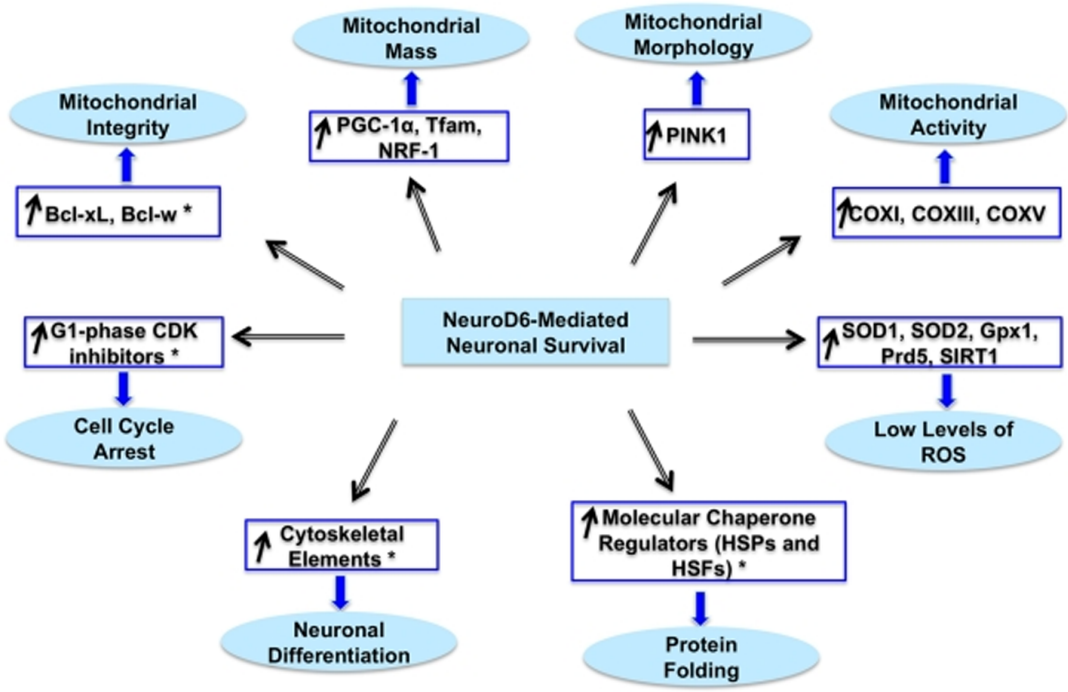

Figure 6-2 Working model of Neurod6-mediated neuronal survival and resistance to oxidative stress in PC12 cells (cited from Uittenbogaard *et al*., 2010)

The timing of onset of Neurod6 expression at E15.5 in VTA neurons coincides with timing of axonal targeting to forebrain structures. This is a less understood energy demanding process in mDA development possibly requiring mitochondrial bioenergy reserves and increased ATP production. There is evidence to support a high demand for ATP during the development of mDA neurons (Pacelli *et al*., 2015), In support of this, prior to cell death of VTA neurons at P2, densitometry of TOM-20 fluorescence reveals a significant reduction in the mitochondrial mass of YFP labelled mDA cells in Neurod6 mutants that no longer express Neurod6 at E18.5 compared to YFP+ cells in controls still expressing Neurod6 (Chapter 4). Reduced mitochondrial mass at this stage could just be a consequence of activation of cell death programs or as a direct result of dysregulation of Neurod6-controlled mitochondrial integrity. If this was the case, Neurod6 would not be the first to promote an anti-oxidative stress modulation of mitochondrial function in post-mitotic differentiated mDA neurons. Lmx1a/b transcription factors also modulate mitochondrial function to promote survival of adult mDA neurons (Doucet-Beaupre *et al*., 2016). Inactivation of Lmx1a/b results in reduced respiratory chain activity, increased oxidative stress, mitochondrial DNA damage and dysfunction resulting in degenerative loss of mature mDA neurons (Doucet-Beaupre *et al*., 2016).

NeuroD6 may be neuroprotective for VTA neurons and could regulate their survival by making them less resistant to mitochondrial oxidative stress via the upregulation of pro-survival and anti-apoptotic factors and mediating an anti-oxidant response.

Overexpression of Neurod6 using a *Cre*-inducible system in differentiated mDA cell cultures from mouse embryonic stem (ES) cells could provide mechanistic insights into Neurod6-mediated mDA neuronal survival in a dopamine context.

6.4 Functional redundancy of Neurod1 in promoting survival of Neurod6+ mDA subset

Neurod6 alone is required for the survival of LSi-projecting mDA neurons, however some *Neurod6*+ neurons still develop normally in *Neurod6* mutant mice and send axons to the LSd (see Chapter 4). Given this partial loss of *Neurod6*+ mDA neurons, I also generated and analysed *Neurod6* and *Neurod1* double mutant embryos and found that *Neurod1* also regulates the survival of the *Neurod6*+ mDA neurons (see Chapter 5).

Functional redundancy of bHLH transcription factors of the the NeuroD family have been documented in other regions of the brain in specific neuronal populations. For example, Previously generated Neurod1/6 double mutant mice provide evidence that Neurod1 and Neurod6 are functionally redundant in the granule cells of the hippocampus (Schwab *et al*., 2000). In the Neurod6/1 double mutants generated in this work, Neurod1-deficiency leads to the elimination of almost all surviving Neurod6+ mDA cells suggesting that Neurod1 is functionally redundant in promoting the survival of a proportion of Neurod6+ mDA neurons, most likely those that project to the LSd.

6.4.1 Neurod1/6 expression and role in survival described for the first time in mDA neurons

This thesis describes for the first time the spatiotemporal expression profiles of

Neurod1, Neurod2 and Neurod6 in the ventral midbrain and demonstrates the role of Neurod6 and Neurod1 in mDA neuronal survival. Previous studies have only documented their expression profiles in cortical, hippocampal, cerebellar regions (Schwab *et al*., 1998; Gao *et al*., 2009; Bormuth *et al*., 2013) of the CNS and others in the PNS.

Neurod2 and Neurod6 share redundant roles in the cortex where they are co-expressed in all post-mitotic projection neurons (Bormuth *et al*., 2013). Majority of callosal projection neurons survive until birth in Neurod2/6 double mutants however their axons stall within the SVZ of the cingulate cortex and fail to turn ventrally towards the midline (Bormuth *et al*., 2013). Neurod2/6 double mutants exhibit absence of cortical fiber tracts, defasiculation of callosal axons and permanent agenesis of the corpus callosum (Bormuth *et al*., 2013) due to changes in differential expression of axon guidance molecules Ctn2 (downregulated in mutants) and Robo1 (upregulated in mutants) of collasal projections (Bormuth *et al*., 2013).

Given these redundant functions of Neurod2/6 in cortical development, I also analysed the expression of Neurod2 in the VTA of Neurod6 control embryos. In contrast to NEUROD1 and Neurod6 (YFP), NEUROD2 was not expressed by mDA neurons at E18.5 and was sporadically expressed in TH-negative (possibly GABAergic) cells in rostral and central midbrain sections.

Neurod1/6 double mutant mice reported previously by Schwab *et al*., (2000) displayed a severe lack of the dentate gyrus which prompted a detailed study of the hippocampus. Defects in the total number of cells within the hilar region and of migratory granule cell precursors was more severe in Neurod1/6 double mutants which exhibited 30% further reduction in hilar region cell numbers compared to

Neurod1 single mutants (Schwab *et al*., 2000). Furthermore, these neurons fail to mature and degenerate as a result of cell death since the number of observed TUNEL+ cells were 7.5-fold higher in the hilar region of Neurod1/6 double mutant hippocampal sections compared to wild-type (Schwab *et al*., 2000).

Neurod6 single mutants have a 30% loss of cells in the central mDA region due to cell death as demonstrated by increased TUNEL+ staining at P2 in the absence of Neurod6. Neurod6+ VTA neurons co-express NEUROD1 in the midbrain of E18.5 embryos. Neurod6/1 double mutants display a nearly complete loss of the Neurod6+ mDA subset. This is in support of partial functional redundancy like that observed in hippocampal granule cells only here Neurod1 may be able to partially compensate for Neurod6 in the remaining 70% of Neurod6+ mDA neurons that usually survive in Neurod6 mutants.

6.4.2 Inactivation of Neurod1 in adult Neurod6+ mDA neurons to assess changes in DA axons to LSd

Since Neurod1;Neurod6 double mutants die shortly after birth, it would be useful to use an inducible gene ablation approach to study the mechanism of functional redundancy of Neurod1 in the Neurod6+ mDA subset. For this purpose, Neurod1flox mice (Goebbels *et al*., 2005) are now being crossed with Neurod6Cre/+;R262RYFP/YFP mice for tamoxifen-induced inactivation of Neurod1 in adult Neurod6+ mDA neurons to further dissect the mechanisms of Neurod1/6-mediated mDA neuronal survival and maintenance. Since in E18.5 embryos, mDA projections to the LS have not yet arrived at this stage, this precluded the analysis of LS-projecting axons in Neurod6/1 double mutants. Using a conditional Neurod1flox

line (Goebbels *et al*., 2005) instead to generate Neurod6;Neurod1 double mutants and inactivating Neurod1 later in Neurod6+ mDA neurons, would enable analysis of the role of Neurod1 in LSd-projecting Neurod6+ VTA neurons.

6.4.3 Investigating Neurod6/1 interactions in mDA domain

Expression analysis by *in situ* hybridisation and antibody labelling shows a much broader expression pattern for Neurod1 which is expressed in all mDA neurons of both the VTA and SNc (Chapter 5). It is unknown whether Neurod1 has functions independent of Neurod6 in mDA neurons outside of the Neurod6 mDA subset in TH+/ Neurod6- neurons. Since Neurod1 has broader expression pattern and is expressed earlier in high levels in immature mDA cells and lower levels in mature mDA cells at E13.5, Neurod1 may be important for the induction of mDA neuron terminal differentiation from immature to mature states.

In support of this, analysis of human umbilical cord of full-term births by immunocytochemistry and western blots revealed an upregulation of TH (mature marker), Nurr1 (both immature and mature marker) and Neurod1 upon mDA neuron induction (Paldino *et al*., 2014). Treatment with forskolin, a natural reagent that activates the adenylyl cyclase enzyme and increases the intracellular levels of second messenger cAMP, resulted in the upregulation of neurotrophin Trk receptors and an increased release of BDNF (Paldino *et al*., 2014). This is the only study to date to mention Neurod1 involvement in mDA neurons.

In addition, Neurod1 is expressed earlier at E13.5 than Neurod6 which is detected in mDA neurons of VTA at E15.5, this could suggest that Neurod1 is upstream of

Neurod6. To test if Neurod1 controls Neurod6 expression in mDA neurons or is required for their survival independent of Neurod6, analysis of Neurod6 mRNA transcript levels in the mDA domain of Neurod1 single mutants by *in situ* hybridisation would reveal either a downregulation of Neurod6 or no change in Neurod6 expression, which could suggest that the two act independently of each other or whether they do indeed interact. Comparing numbers of Neurod6+ mDA cells in Neurod1 single mutants would give further insights into Neurod6-independent Neurod1-mediated survival of Neurod6+ mDA neurons.

6.4.4 Comparisons to other transcription factor pairs in mDA development

In contrast to many of the other transcription factor pairs that are required for mDA development such as Foxa1/2, Lmx1a/b and En1/2, results suggest Neurod1/6 only act together in post-mitotic mature mDA neurons. Foxa1/2 for example, are required at multiple stages of mDA development for the specification of mDA progenitors, the early differentiation into immature and late differentiation into mature mDA neurons (Ferri *et al*., 2007). This requirement for Foxa genes is dosage-dependent since higher concentrations (more allelic copies) of Foxa1/2 are required at later stages of mDA development and therefore the timing and duration of mDA differentiation is regulated by Foxa1/2 (Ferri *et al*., 2007).

In contrast, the effects of Neurod6 and Neurod1 seem late acting on differentiated mDA neurons. Considering Neurod6;Neurod1 mutant analysis of TH+/YFP+ cell numbers at E18.5 (Chapter 5) shows proportional changes in the percentage of TH+/YFP+ cells with varying allelic-inactivation combinations, raises speculation that Neurod6 and Neurod1 may regulate mDA neuron survival in a gene dosage-

dependent manner. Several other transcription factor pairs regulate mDA survival including Foxa1/2, Lmx1a/b, En1/2 and now Neurod1/6.

6.5 Towards understanding behaviour: Functional implications for Neurod6+ VTA subset

Neurod6+ mDA neurons identify a novel subset in the VTA characterised by the expression of unique molecular markers and the innervation of these neurons on to the LS in the forebrain. The role of Neurod6 itself in these mDA neurons is that of survival. However, the functional relevance of this Neurod6-defined VTA subset of DA neurons remains to be uncovered. Assessing the function of this population would enhance the significance of this work.

Although the selective expression of Neurod6-Cre reported here will likely become a highly effective tool to characterise subsets of DA neurons in the VTA and their physiological function, this mouse line was also a major limitation in elucidating the function of this Neurod6+ mDA subset of neurons in a behavioural context. This is because in Neurod6 mutants, the absence of Neurod6 is not unique to mDA neurons but Neurod6 expression is absent from all other brain regions where Neurod6 would usually be expressed including the cortex, hippocampus, hypothalamus, and cerebellum (Goebbels *et al*., 2006). This thereby precluded the behavioural analysis of Neurod6 mutants to determine the functional relevance of Neurod6+ mDA neurons that project to the LS.

6.5.1 Viral and genetic approaches for future behavioural studies

To study the function of mDA-specific Neurod6+ neurons, injections of Cre-dependent Designer Receptor Exclusively Activated by Designer Drugs (DREADDs)

in the VTA region for either activation of inactivation of these neurons in *Neurod6*$^{Cre/+;}$*R26R*$^{YFP/YFP}$ mice or the use of *Cre*-dependent channel rhodopsin and optogenetics of these neurons could be employed in future work. Alternatively, dual-recombinase genetic intersectional approaches also allow for high selectivity for labelling DA subsets (reviewed in Dymecki *et al*., 2010). In a dual-recombinase approach, two site-specific recombinases rather than one are required to activate a reporter transgene (Dymecki *et al*., 2010) and only the cells contained in the overlap between expression patterns driven by the Cre and the Flpe transgenes are selected for fate mapping (Dymecki *et al*., 2010). This approach would enable the detection of the 'activated' dual-recombined constitutively expressed GFP reporter transgene as the double-positive (for example, TH+/NeuroD6+) cells.

6.5.2 Functional relevance for LS-projecting Neurod6+ mDA subset in stressful, social and addictive behaviours

Limited studies have reported observations of elevated extracellular DA levels and increased DA releasability in the LS as a result of the actions of several drugs. The co-release of GABA and DA from non-canonical synthesis of GABA (Kim *et al*., 2015) in Aldh1a1+/Neurod6+ VTA neurons should also be explored in the context of alcohol consumption and dependency. Future work should explore behaviours relevant to: (i) Addiction particularly those linked to amphetamine (Renard *et al*., 2014), cocaine (Reddy *et al*., 2016), morphine self-administration (Le Merrer *et al*., 2007), and alcohol consumption (Kim *et al*., 2015), (ii) Sensory information processing of stress (Adams and Moghaddam, 2000) or of (iii) septo-hippocampal modulation and learning (Robinson *et al*., 1979; Yamamuro *et al*., 1995).

6.6 Final Remarks

The understanding of heterogeneity of mDA neurons particularly of VTA-specific molecular subsets links to wider implications of the molecular identities of DA neurons to reward-based behaviours and several clinical conditions in addiction, social stress and depressive states. Dissecting molecular subsets of VTA neurons also contributes towards building our understanding of neuronal survival and neuroprotective mechanisms in mDA neurons. It is well established that VTA neurons are more protected in neurodegeneration. In PD, SNc neurons are highly susceptible to neuronal death compared to VTA neurons that are clearly more resilient. Unravelling the factors that constitute VTA mDA neurons more resilient to cell death by regulation of neuronal survival via mitochondrial pathways, activation of anti-apoptotic programs and protection against oxidative stress during their development, will aid our understanding of therapeutic targets and requirements for mDA neuron survival and regeneration in the treatment of DA-related neurodegenerative and neuropsychiatric disorders. Furthermore, dissecting unique and overlapping DA circuit pathways by genetic tools that label individual DA populations at the molecular level will aid discrimination of limbic system psychiatric behavioural conditions that present with very similar symptoms. Together, these could aid not just therapy towards reward-based DA functions of the limbic system but also perhaps cell-based therapy for mDA protection in neurodegeneration. For these reasons, mDA heterogeneity is an important and expanding field and dissecting complexity of dynamic subsets and their projection patterns work towards a bigger picture of how DA neurons in the brain regulate human physiological, behavioural and psychological functions.

Chapter 7. Appendix

7.1 The levels, expression profiles and functions of DA receptors in the brain (summarised from Beaulieu and Gainetdinov, 2011)

DA receptor subtype:	*Level of expression:*	*Brain areas expressed in:*	*Functions:*
D_1	High	nigrostriatal, mesolimbic, and mesocortical areas, such as the caudate-putamen (striatum), NAc, SN, olfactory bulb, amygdala, and frontal cortex	locomotor activity, reward and reinforcement, learning and memory
	Low	hippocampus, cerebellum, thalamic areas, and hypothalamic areas	
D_5	Low	pyramidal neurons of PFC, the premotor cortex, cingulated cortex, entorhinal cortex, SN, hypothalamus, the hippocampus, and dentate gyrus	cognitive functions
	Very low	Medium spiny neurons (GABAergic) of the caudate nucleus and NAc	
D_2	High	striatum, NAc, the OT, SN, VTA, hypothalamus, cortical areas, septum, amygdala, and hippocampus	locomotor activity, reward and reinforcement, learning and memory
D_3	High	limbic areas, such as in the shell of the NAc, the OT, and the islands of Calleja	locomotor activity, reward and reinforcement, cognitive functions
	Low	striatum, the SNpc, VTA, hippocampus, the septal area, and in various cortical areas	
D_4	Lowest	frontal cortex, amygdala, hippocampus, hypothalamus, globus pallidus, SNr and thalamus	cognitive functions

7.2 Human proteins with basic helix-loop-helix DNA binding domain

AHR
AHRR
ARNT
ARNT2
ARNTL
ARNTL2
ASCL1
ASCL2
ASCL3
ASCL4
ATOH1
ATOH7
ATOH8
BHLHB2
BHLHB3
BHLHB4
BHLHB5
BHLHB8
CLOCK
EPAS1
FERD3L
FIGLA
HAND1
HAND2
HES1
HES2
HES3
HES4
HES5
HES6
HES7
HEY1
HEY2
HIF1A
ID1
ID2
ID3
ID4
KIAA2018
LYL1
MASH1
MATH2
MAX
MESP1
MESP2
MIST1
MITF
MLX
MLXIP
MLXIPL
MNT
MSC
MSGN1
MXD1
MXD3
MXD4
MXI1
MYC
MYCL1
MYCL2
MYCN
MYF5
MYF6
MYOD1
MYOG
NCOA1
NCOA3
NEUROD1
NEUROD2
NEUROD4
NEUROD6
NEUROG1
NEUROG2
NEUROG3
NHLH1
NHLH2
NPAS1
NPAS2
NPAS3
OAF1
OLIG1
OLIG2
OLIG3
PTF1A
SCL
SCXB
SIM1
SIM2
SOHLH1
SOHLH2
SREBF1
SREBF2
TAL1
TAL2
TCF12
TCF15
TCF21
TCF3
TCF4
TCFL5
TFAP4
TFE3
TFEB
TFEC
TWIST1
TWIST2
USF1
USF2

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